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Characterization of Linear Chromosome Segregation and Condensation of Streptomyces coelicolor

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CHARACTERIZATION OF LINEAR CHROMOSOME SEGREGATION AND CONDENSATION OF *STREPTOMYCES COELICOLOR*

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ABSTRACT

CHARACTERIZATION OF LINEAR CHROMOSOME SEGREGATION AND CONDENSATION OF *STREPTOMYCES COELICOLOR*

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DNA segregation and condensation must occur accurately during cell division to ensure the survival of daughter cells. Most prokaryotes have a single, circular genome, which is simultaneously replicated, segregated and condensed during cell division. *Streptomyces coelicolor* is a sporulating, filamentous bacterium with a large, linear genome. Syncytial aerial hyphae contain numerous copies of the genome, which must be synchronously segregated into prespore compartments while up to 100 septa form. The proteins that control and accomplish this complicated process are of interest. Genetic analysis revealed that *S. coelicolor* can survive without three DNA segregation proteins, SMC, FtsK and ParB, which normally result in synthetic lethal phenotypes in unicellular bacteria. A Δ*smc* Δ*ftsK* Δ*parB* triple mutant was still able to segregate genetic material to 90% of its spores, but exhibited a fourfold decrease in viability when compared to wild type. For *S. coelicolor*, there must be considerable redundancy in genome segregation to

overcome the loss of these genes. Furthermore, the large genome of *S. coelicolor* has to be properly condensed in order for it to fit inside a spore which is 1 μm in length. Genetic analysis of *scpA* and *scpB*, the gene products are thought to be involved in DNA condensation by interaction with SMC, revealed that neither were required for viability, but produced spores with a bilobed DNA architecture, unlike wild type or Δ*smc* mutant spores. It was concluded that this morphological phenotype was not the result of an interaction of the *scpAB* with *smc*, as the *smc* mutant did not present this phenotype. This bilobed nature of the *scp* mutants prompted the investigation of spore ploidy. Using several different but complementary methods, evidence was obtained suggesting that *S. coelicolor* spores and that of other species are diploid. A specific and dynamic movement of the origin of replication was discovered very late in spore development and a novel gene, *parA2*, was found to directly or indirectly play a role in this process. Together, these data reveal new information in order to better understand how linear chromosomal DNA is segregated, condensed and localized in this sporulating bacterium.

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ATTRIBUTIONS

Hans Wildshutte was the first to observe that a *Streptomyces coelicolor smc* deletion strain was viable. He constructed the *smc* complementation plasmid, pHW35, used in Chapter 2.

Joseph McCormick constructed the *smc* and *ftsK* deletion strains, HJ2 and JRM148, respectively, used in Chapter 2.

Aimee Belanger constructed plasmids pAEB228 and pAEB232, used to make *ftsK* deletion strain RMD24, used in Chapter 2.

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Introduction

Prokaryotic DNA Replication & Orientation

The circular prokaryotic chromosome replicates bi-directionally starting at *oriC*, which is localized near the center of an elongating cell in rod-shaped bacteria. Each half of the chromosome is duplicated by different replication forks that end at the terminus opposite of *oriC* to complete replication (Sherratt, 2003; Wu, 2004; Gitai *et al*., 2005). The origin regions of the duplicated chromosome are the first to separate from one another by moving to the ¼ and ¾ positions of the elongating cell (For review see Lemon & Grossman, 2000). The left and right arms of the chromosome, located between *oriC* and *ter*, are organized on either side of the cell center (Dingwall & Shapiro, 1989; Teleman *et al*., 1998; Niki *et al*., 2000; Marczynski & Shapiro, 2002; Nielsen *et al*., 2006). How this mechanism of DNA arrangement concomitantly occurs during replication is still unknown.

E. coli, a Gram negative bacterium, divides by binary fission. In this organism, newly replicated DNA was visualized using [^{3}H] thymidine incorporation, which indicated that DNA replication occurs at mid cell (Koppes *et al*., 1999). In newborn cells, the origin is located at mid cell; however, once replication begins, the newly duplicated origins move to the quarter cell positions (Wang *et al*., 2005; Adachi *et al*., 2005; Bates & Kleckner, 2005). During replication of the chromosome, loci between the *oriC* and *ter* are found to occupy specific regions in the new daughter cells (Niki *et al*.,

2000). Fluorescent reporter-operator systems (FROS) demonstrated that new sister chromosomes have a distinct symmetry in the elongating *E. coli* cell (Wang *et al*., 2006).

B. subtilis is a sporulating, Gram positive bacterium that also divides by binary fission. Sporulation-associated division is positioned asymmetrically, resulting in a large mother cell and a smaller forespore. Although the vegetative growth and sporulation portions of the lifecycle are different, the division machinery required for each is similar (Errington *et al*., 2003). At the onset of sporulation and during vegetative growth, *oriC* is located near both poles of the cell after replication of the DNA (Wu & Errington, 1994; Glaser *et al*., 1997; Lin *et al*., 1997; Webb *et al*., 1997). Replication is thought to occur at mid cell, because components of the replisome were visualized *in vivo* at mid-cell (Lemon & Grossman, 1998; Imai *et al*., 2000; Meile *et al*., 2006; Berkmen & Grossman, 2006). After the initiation of DNA replication, the origins of replication move to opposite poles of the cell (Glaser *et al*., 1997; Sharpe & Errington, 1998; Teleman *et al*., 1998; Webb *et al*., 1998).

The Gram negative *C. crescentus* asymmetrically divides to produce a swarmer and a stalked cell. DNA replication only occurs in the stalked cell where it initiates only once per cell cycle (Degnen & Newton, 1972). In the non-replicative swarmer cell, *oriC* is positioned at the same pole as the pili and flagellum (Viollier *et al*., 2004). The DNA replication apparatus is not stationary, as it is in *B. subtilis* (Jensen *et al*., 2001). The replisome is located at the stalked pole when replication begins and is passively displaced to midcell most likely by the newly replicated DNA (Jensen *et al*., 2001). Although the replisome is untethered, newly replicated origins are still rapidly moved to opposite poles of the cell (Jensen & Shapiro, 1999). The loci between the *oriC* and *ter* regions of the

chromosome were found to move in an organized fashion (Viollier *et al*., 2004). They have a specific location in each daughter cell, ordered linearly according to the genetic map (Viollier *et al*., 2004).

Prokaryotic DNA Segregation

In the unicellular bacteria studied thus far, DNA segregation proteins are typically conserved. Although their functions and requirements for viability may be different, most bacteria have homologues of known DNA segregation proteins.

FtsK

FtsK is a member of the FtsK/SpoIIIE/Tra family of proteins. The $FtsK_N$ (N-terminal domain) is the least conserved region of the protein and it is predicted to contain either four or five transmembrane segments, depending on the bacterium. The $FtsK_N$ is separated from $FtsK_C$ (C-terminal domain) by a linker domain. The cytoplasmic $FtsK_C$ is a molecular motor, which translocates DNA at rates up to 7 kb s^{-1} and is thought to be involved in preventing chromosomal DNA trapping by the invaginating septum during DNA segregation and division (Saleh *et al*., 2004; Pease *et al*., 2005). There are three separate domains of $FtsK_C$ based on the solved crystal structure of *Pseudomonas aeruginosa* FtsK: α, β, and γ (Massey *et al*., 2006). The α and β domains oligomerize to form a ring that accommodates double-stranded DNA and contain the core ATPase machinery (Massey *et al*., 2006; Yates *et al*., 2003). The γ domain interacts with the site specific recombination machinery to resolve dimers and recognizes FtsK orienting polar sequences (KOPS) within the DNA (Yates *et al*., 2003 & 2006; Pease *et al*., 2005; Bigot *et al*., 2005; Sivanathan *et al*., 2006; Löwe *et al*., 2008).

Generally, this protein has several functions. It is involved in chromosome decatenation, DNA segregation, and cell division. In *E. coli*, FtsK has been shown to physically and functionally interact with topoisomerase IV, the chromosomal decatenase encoded by *parC* and *parE* (Espeli *et al*., 2003). In this study, topoisomerase IV was recovered in immunoprecipiates of FtsK and two-hybrid analysis indicated that the interaction was mediated by the ParC subunit of topoisomerase IV (Espeli *et al*., 2003). The ParC subunit contains the DNA binding and cleavage domains of topoisomerase IV (Peng & Marians, 1993). Topoisomerase is localized at the cell center, where it decatenates chromosomes after they are replicated at midcell; the $FtsK_N$ serves as a membrane anchor to secure the protein at the septal ring (Espeli & Marians, 2004; Wang *et al*. 2005). Interestingly, a recent paper suggests that chromosome unlinking can also be achieved in cells lacking topoisomerase IV, by site specific recombination using XerCD-*dif* (Grainge *et al*., 2007). $FtsK_C$ is required for XerCD resolution of chromosome dimers at *dif* sites (Steiner, 1999).

Whether the function of FtsK is essential varies in prokaryotic organisms (Table 1.1). A complete *ftsK* deletion in *E. coli* results in cell death (Begg *et al*., 1995). $FtsK_N$ is predicted to span the membrane four times and is required for its proper localization and cell division (Wang & Lutkenhaus, 1998; Dorazi and Dewar, 2000). This phenotype can be suppressed by the overexpression of other cell division proteins, such as FtsQ, FtsA or FtsZ, indicating that the function of FtsK is not novel (Geissler & Margolin, 2005). On the other hand, insertion mutations in the 3' end of *ftsK* are not lethal, but can result in the formation of smooth filaments (Begg *et al*., 1995; Diez *et al*., 1997; Draper *et al*., 1998; Yu *et al*., 1998).

In *B. subtilis*, the FtsK homolog (SpoIIIE) is dispensable for vegetative growth, but essential for sporulation (Wu & Errington, 1997). SpoIIIE functions to clear DNA from the division septa during vegetative growth (Sharpe & Errington, 1995; Britton & Grossman, 1999; Lemon & Grossman, 2001). During sporulation, division occurs asymmetrically; the septum is formed near one pole and 30% of one chromosome is trapped within the forespore (Levin & Losick, 1996; Pogliano *et al*., 2002). It has been proposed that SpoIIIE travels to this septum and translocates the trapped genetic information from the mother cell to the forespore compartment (Wu & Errington, 1997; Bath *et al*., 2000; Chary & Piggot, 2003; Liu *et al*., 2006). SpoIIIE recognition sequences (SRS), like KOPS, were found to be necessary for directional DNA translocation (Ptacin *et al*., 2008). The SRS motif, GAG(C/A)AGGG, is similar to the KOP motif, GGGNAGGG (Bigot *et al*., 2005; Ptacin *et al*., 2008).

Similar to FtsK in *E. coli*, in *C. crescentus*, the first 258 amino acids were found to be essential for localization of FtsK to the division site (Wang *et al.,* 2006). The localization of FtsZ was also dependent on $FtsK_N$ in *C. crescentus* (Wang *et al.,* 2006). In contrast, FtsZ does not require FtsK/SpoIIE for localization in *E. coli* and *B. subtilis*, respectively (Chen & Beckwith, 2001; Wang & Lutkenhaus, 1998). Because $FtsK_C$ contains the motor domain, which is thought to play a role in segregating the DNA, a C-terminal depletion mutant was constructed and 15-20% of cells displayed defects in DNA segregation; DNA was distributed unevenly (Wang *et al.,* 2006). It is clear that *C. crescentus* FtsK is crucial not only for proper segregation of the chromosome, but also for division (Wang *et al*., 2006).

Table 1.1: The effect of *ftsK/spoIIIE* mutations for different unicellular bacteria.

	Essential for viability	**C-terminus**	**N-terminus**
E. coli	Yes	not required for viability; smooth filaments form	required for viability; localization to the septa
B. subtilis	No	required for DNA transfer during sporulation	required for targeting to the sporulation septum
C. crescentus	Yes	Required for viability; segregation of DNA	required for viability; localization to the septum

ParAB and *parS* Sites

Partitioning of the chromosome has been studied by analyzing the *par* genes, which were first discovered on low-copy number plasmids in *E. coli* (Austin & Abeles, 1983; Ogura & Hiraga, 1983; Gerdes *et al.,* 1985). Three loci are found in most plasmids with this type of partition system: two *trans*-acting proteins (ParA/SopA and ParB/SopB) and a *cis*-acting site (*parS*). In *E. coli* plasmids P1 and F, ParB/SopB is a DNA-binding protein that binds to centromere-like *parS* sites on the plasmid. Plasmid segregation is thought to be aided by the ParA/SopA Walker-type ATPase activity resulting from the interaction of ParA/SopA with the ParB/SopB-*parS* nucleoprotein complex (Bouet & Funnell, 1999; Yates *et al.,* 1999). Recent experiments have suggested that ATP is not needed for SopA binding to specific operator DNA sequences, but actually stimulates its binding to non-specific DNA (Bouet *et al*., 2007). Castaing *et al*., (2008) completed experiments to identify specific amino acids required for this SopA-non-specific DNA interaction. Specifically, a lysine to alanine amino acid change in codon 340 reduced ATP-dependent DNA binding and was 100-fold less accurate at partitioning mini-F plasmids than wildtype (Castaing *et al*., 2008). This suggests that the lysine in this location is important not only for DNA binding, but also plasmid partitioning.

B. subtilis has a chromosomal *par* system. The ParA and ParB homologs are called Soj and Spo0J, respectively. Spo0J binds to at least eight *parS* sites within 20° of *oriC*, which is located at 0° of the circular chromosome (Glaser *et al*., 1997; Lin *et al*., 1997; Lin & Grossman, 1998). Spo0J was recently found to spread along the DNA, with *parS* sites located >1kb from *oriC* (Breier & Grossman, 2007). An Δ*spo0J* mutant results in an increased number of anucleate vegetative cells (1-2% anucleate compared to 0.02% for wild type), indicating a role in partitioning the chromosome (Ireton *et al*., 1994; Lin *et al*., 1998). On the other hand, an Δ*soj* mutant has only minor effects on chromosome segregation, which indicates that Soj does not play a major role in this process (Marston & Errington, 1999). Spo0J and Soj interact and it was suggested that the formation of a Soj dimer is a prerequisite for this interaction (Marston & Errington, 1999; Leonard *et al*., 2005a, b). Interestingly, conserved, surface-exposed arginines in Soj were found to be important to efficiently partition plasmids, suggesting a role for this area of the protein in DNA binding (Hester & Lutkenhaus, 2007). At the start of sporulation, *oriC* was not correctly positioned in a Δ*spo0J* Δ*soj* strain, suggesting that Soj and Spo0J are needed for correct chromosome orientation during sporulation (Sharpe & Errington, 1996). Recently, two-hybrid analysis was used to show that Soj may control the initiation of DNA replication by a direct interaction with DnaA, the protein which recognizes *oriC* and initiates DNA replication (Murray & Errington, 2008).

C. crescentus also has a chromosomal *par* system in which ParB binds to approximately six *parS* sites within 20 kb of *oriC* (Easter, Mohl & Gober, unpublished data as cited by Mohl *et al*., 2001). After DNA replication, ParB localizes to the poles of the cell, which supports *oriC* movement to the poles (Mohl & Gober, 1997). The

C. crescentus Par proteins are essential for cell viability (Mohl & Gober, 1997). ParA-ATP promotes dissociation of the ParB-*parS* complex (Easter & Gober, 2002). Overexpression of ParA results in a filamentous phenotype, which indicates that the ratio of ParA and ParB within the cell is important for cell division (Mohl *et al*., 2001; Easter & Gober, 2002). This is similar to what was found for P1 plasmids of *E. coli*, where the position of ParB depended on ParA (Erdmann *et al*., 1999). Therefore, the concentration of ParA and ParB serves a particular purpose in these bacteria.

The *E. coli* chromosome does not have homologues of the Par system. Instead, *migS* has been identified as the centromere-like element for bipolar positioning of *oriC* (Yamaichi & Niki, 2004; Fekete & Chattoraj, 2005). The 25 bp *migS* sequence is located 211 kb from *oriC* in the clockwise direction and is unique to the *E. coli* genome (Yamaichi & Niki, 2004). When *migS* was disrupted, *oriC* positioning still occurred at mid-cell just prior to replication, suggesting that *migS* does not play a role in tethering the chromosome to mid-cell, but the distribution of the origins after replication was disturbed (Yamaichi & Niki, 2004). Although *migS* was found to play a role in bipolar positioning of *oriC*, it is not required for proper chromosome segregation in dividing cells (Yamaichi & Niki, 2004).

Structural Maintenance of Chromosomes

Homologs of structural maintenance of chromosome (*smc)* genes are present in most archaea, prokaryotes and eukaryotes and they are believed to function in chromosome maintenance and structure (Hirano, 1999; Jensen & Shapiro, 1999; Koshland & Strunnikov, 1996). SMC consists of an N-terminal nucleotide triphosphate-binding domain, a central domain containing two long segments of coiled-coil motif

separated by a hinge region, and a C-terminal domain (Melby *et al.*, 1998). In *E. coli*, the functional homolog of SMC is MukB. A *mukB* mutation resulted in temperature-sensitive growth, anucleate cells (5% when cells were grown at 22°C and up to 20% at 42°C) and unorganized nucleoids (Niki *et al*., 1991). Eukaryotic SMCs, *B. subtilis* SMC and the *E. coli* MukB proteins function in complexes, which are thought to organize and compact the chromosome (Table 2) (Yamazoe *et al*., 1999; Cobbe & Heck, 2000; Ohsumi *et al*., 2001; Hirano, 2002; Soppa *et al.,* 2002; Mascarenhas *et al*., 2002).

MukB forms a complex *in vitro* with MukF and MukE (Yamazoe *et al*., 1999). It was shown that MukF and MukB can form a complex in the absence of MukE, but MukF is essential for the interaction between MukB and MukE. MukB-GFP localization was found to be aberrant when either MukF or MukE is absent, suggesting that the complex functions as a unit *in vivo* (Ohsumi *et al*., 2001). MukB is thought to be a macromolecular clamp that efficiently compacts DNA both in the absence of ATP and MukEF (Cui *et al*., 2008). ATP stimulates the *in vitro* initiation of MukB-dependent condensation, but not the propagation of this process (Cui *et al*., 2008). Using a MukB-GFP fusion and FROS observe *oriC*, it was found that MukB colocalizes thoughtout the cell cycle with *oriC* (Danilova *et al*., 2007).

In *B. subtilis,* an *smc*-null mutant resulted in decondensed nucleoids and anucleate cells (Britton *et al*., 1998). A *smc spoIIIE* double mutant in *B. subtilis* resulted in a synthetic lethal phenotype (Britton & Grossman, 1999). It has been shown that SMC in *B. subtilis* has at least two interacting proteins, ScpA and ScpB (segregation and condensation proteins) (Soppa *et al.,* 2002) (Table 1.2). This interaction was proven by yeast two hybrid analysis and immunoprecipitation (Mascarenhas *et al*., 2002). ScpA

and ScpB localize to mid cell early in the cell cycle and then follow the movement of *oriC* during DNA replication (Mascarenhas *et al*., 2002). Mutations that disrupt either *scpA* or *scpB* lead to temperature-sensitive growth, 10% anucleate cells and loss of chromosome structure, a phenotype that resembles the *smc* deletion strain (Mascarenhas *et al*., 2002; Graumann, 2001) (Table 1.2). Sequence similarity between ScpA and MukF and ScpB and MukE could not be found, but all are small soluble proteins with many alpha-helixes in their secondary structures (Soppa *et al*., 2002).

Homologs for both *scpA* and *scpB* (CC2004 and CC2005) exist in *C. crescentus*, but an SMC-ScpA-ScpB complex has yet to be studied (Table 1.2). In *C. crescentus,* an *smc*-null mutation causes defects in nucleoid structure and a conditional lethal phenotype, leading to cell cycle arrest before division can take place (Jensen & Shapiro, 1999). This result suggested that SMC may play a role in a cell cycle checkpoint. SMC was found to localize in a punctate pattern throughout the *C. crescentus* life cycle using immunofluorescence microscopy (Jensen & Shapiro, 2003). A subpopulation of predivisional cells was found to have SMC localized at the poles of the cell, indicating protein aggregation. These polar foci are lost when cell division initiates, suggesting that the location and aggregation of SMC changes as the cell cycle progresses in *C. crescentus*.

Table 1.2: The effect of *mukB* or *smc* mutations on bacterial species and their protein partners.

	Δ*mukB* or Δ*smc*	**Interactions**
E. coli	unorganized nucleoids; anucleate cells; ts growth	MukEF
B. subtilis	decondensed nucleoids; anucleate cells	ScpAB
C. crescentus	cell cycle arrest	untested

An Introduction to *Streptomyces coelicolor*

S. coelicolor is a Gram-positive, soil-dwelling bacterium that belongs to the group of bacteria responsible for producing many of the world's natural antibiotics and other biologically active compounds. This streptomycete is filamentous and has an unusual lifecycle. It resembles certain filamentous fungi in that it has both vegetative, non-reproductive growth, as well as aerial, reproductive growth. To begin the life cycle of *S. coelicolor*, a spore germinates and forms a network of branching vegetative hyphae, where DNA is not condensed and septation is infrequent and not essential (for review see Flärdh, 2003). Vegetative filaments give rise to aerial filaments that grow away from the colony surface. Within a given multi-genomic hypha, septation occurs synchronously to form a chain of spores (Wildermuth & Hopwood, 1965; Schwedock *et al*., 1997; Flärdh *et al*., 1999).

The 8.67 Mbp linear genome of *S. coelicolor* strain A3(2) has been sequenced (Bentley *et al*., 2002). The genome is 72.12% G-C, with a centrally located *oriC* region and terminal inverted repeats located at the ends of the chromosome (Zakrzewska-Czerwinska & Schrempf, 1992; Musialowski *et al*., 1994). *S. coelicolor* normally has two plasmids, which have also been sequenced (Bentley *et al*., 2004; Huag *et al*., 2003).

The linear plasmid SCP1 is 365 kb and the circular plasmid SCP2 is 31 kb. These plasmids are not required for propagation of *S. coelicolor*.

DNA Replication and Orientation

Replication of the *S. coelicolor* chromosome lasts 90 to 100 minutes at 30°C (Kornberg & Baker, 1992). When and where replication occurs in this multinucleoid prokaryotic has not been thoroughly studied. *S. coelicolor* DNA replication was first examined in early vegetative filaments where it was found to take place in non-specific regions (Kummer & Kretschmer, 1986). Twenty years later, an EGFP fusion to DnaN, was used to visualize the replication machinery in vegetative and aerial filaments of *S. coelicolor*. DnaN forms the sliding β clamp that attaches DNA polymerase III to the template to enhance processivity. Using this fusion protein, non-synchronous chromosome replication was observed in vegetative hyphae (Ruban-Osmialowska *et al*., 2006). In that study, 60% of the vegetative hyphae analyzed had diffuse DnaN-EGFP foci dispersed throughout the entire filament. In hyphae containing bright DnaN-EGFP foci, the foci were found to have variable spacing, indicating no defined coordination of DNA replication. Consistent with this data, Yang and Losick (2001) found no regular distribution of *oriC* foci in vegetative hyphae of *S. coelicolor* using FISH analysis. Because cell wall growth in vegetative filaments is known to occur mostly at the tips (Flärdh, 2003), it was once thought that this region could be a major site of DNA replication (Gray *et al*., 1990; Yang & Losick, 2001). Ruban-Osmialowska *et al*. (2006) addressed this idea and found that there was no difference with the number of DnaN-EGFP foci, or their spacing, between the tips and the remainder of the vegetative filaments. Combined, the interpretation of this information would be that vegetative

hyphae do not have distinct regions where DNA replication takes place or a regular, organized placement of the chromosomes within the filaments.

DNA replication in aerial filaments, visualized by DnaN-EGFP, was also found to be unorganized. Sixty-eight percent of the apical compartments of aerial filaments were found to have bright or bright and diffuse DnaN-EGFP foci, indicating active DNA replication. This mixture of bright and diffuse foci suggests that DNA replication within individual aerial filaments is asynchronous. Also, the number of foci in both vegetative and aerial filaments was substantially less than the required chromosome number (Ruban-Osmialowska *et al*., 2006). This suggests that perhaps two or more chromosomes were being replicated at each DnaN-EGFP focus. We belive that following vegetative replication, one or two chromosomes are partitioned into nascent aerial filament, where they replicate to produce enough chromosomes for each spore compartment in that particular filament. There seems to be no overt suggestion of chromosome organization within these filaments until the stage when segregation of the DNA into prespore compartments occurs. Although EGFP fusions are often used to determine the subcellular location of proteins, this method does have its limitations. The EGFP is usually attached to either the C- or N-terminal domain of the protein of interest, where it can be easily removed by proteolysis. This was not the case for the DnaN-EGFP fusion, which proved to be functional *in vivo* (Ruban-Osmialowska *et al*., 2006).

DNA Segregation

In unicellular bacteria, newly replicated *oriC* regions of the chromosome are moved to the poles, or quarter positions, in order to ensure that each daughter cell receives a copy of the chromosome. In differentiating aerial hyphae, *S. coelicolor* must

correctly position, segregate and condense dozens of chromosomes synchronously, as the septa invaginate in concert. This enormous task must be completed accurately so that each prespore compartment receives genetic material. Exactly how *S. coelicolor* simultaneously coordinates all of these processes is unknown. These mechanisms in this organism are extremely accurate, however, with a wild type strain producing less than 1% anucleate spores. The proteins that play a role in this complicated process are still being discovered, but much progress has been made in defining the contributions of FtsK, ParA and ParB homologs (Wang *et al*., 2007; Ausmees *et al*., 2007; Kim *et al*., 2000).

Macroscopically, *ftsK*-null mutants were found to form more heterogeneous colonies than the wild type *S. coelicolor* strain (Wang *et al*., 2007; Ausmees *et al*., 2007). Wang *et al*. (2007) found that these abnormal colonies were due to large deletions at the ends of the chromosome, as visualized by PCR, which may occur due to the potential for the chromosomal ends to be trapped in the sporulation septa. Because nucleoid occlusion does not occur in *S. coelicolor* (Flärdh, 2003), the septa can guillotine the chromosome, thereby producing chromosome deletions. This suggests that FtsK plays a role in segregating the chromosome during sporulation. *S. coelicolor* FtsK was found to localize at the sporulation septa, as expected from research in unicellular bacteria (Wang *et al*., 2007; Ausmees *et al*., 2007). An FtsK-EGFP fusion revealed that FtsK was present during the reproductive phase of the life cycle and appeared to be distinctly organized in aerial filaments at the sporulation septa, which again suggested that the role of this protein was required during sporulation (Wang *et al*., 2007; Ausmees *et al*., 2007).

Two additional proteins which are similar to that of the SpoIIIE/FtsK family of proteins are SmeA (small putative membrane protein) and SffA (SpoIIIE/FtsK-family

protein), which are expressed during development (Ausmees *et al*., 2007). *smeA* encodes a 63 amino acid protein that contains a single putative transmembrane domain. *sffA* encodes a 532 amino acid protein with an N-terminal transmembrane domain and a C-terminal ATPase domain resembling that of the FtsK family of DNA motor proteins. Macroscopically, wild type spores produce a gray pigment when they are fully developed, but the Δ*smeA* mutant produced light gray colonies, when compared to wild type, indicating a role for this gene product in spore formation (Ausmees *et al*., 2007). Microscopically, a Δ*smeA* Δ*sffA* double mutant revealed that DNA was more diffuse in sporulating hyphae, when compared to wild type, and the number of anucleate spores increased (from 0.1% in wild type to 0.6% in the double mutant), which suggests a role for these proteins in DNA condensation and segregation. This study found that the spore cell wall of the double mutant was thinner than wild type and they were more susceptible to heat inactivation. These proteins also appeared to be required for the separation of spores, as spores were still seen in chains after 8 days of growth, in contrast to the aerial mycelium of wild type, which has free spores at this time. The phenotype is pleiotropic, which indicates that SmeA and SffA may have multiple functions in *S. coelicolor*, such as spore maturation, DNA segregation and condensation, spore separation and pigmentation, or that it regulates checkpoints that block downstream events from happening. Nonetheless, the double mutant strain is viable suggesting that other proteins ensure proper synchronous segregation of multiple chromosomes along an aerial filament.

S. coelicolor contains a chromosomal Par system, which functions in partitioning the chromosomes during sporulation. ParB binds about 20 *parS* sites and spreads over a

200 kb segment centered on *oriC* in the *S. coelicolor* chromosome (Jakimowicz *et al*., 2002). ParB-EGFP foci are irregularly spaced in vegetative hyphae and early aerial filaments (Jakimowicz *et al*., 2005). In late aerial filaments, ParB foci are regularly spaced along the axis of the filament before DNA segregation and septation occur (Jakimowicz *et al*., 2005). Deletion of *parB*, or of the *parAB* operon, results in 15% anucleate spores, suggesting a role in DNA segregation (Kim *et al*., 2000). Perhaps ParB positions the region of the chromosome containing *oriC*, preparing and actively participating in its faithful segregation into prespore compartments. An *S. coelicolor* *parA*-null mutant had chromosome segregation defects (27% anucleate), suggesting that ParA may be involved in the formation or activity of ParB complexes (Jakimowicz *et al*., 2007). It was found that ATP binding to ParA was critical fro ParA dimerization and for its interaction with ParB (Jakimowicz *et al*., 2007). Although, immunofluorescence microscopy indicated that ParA forms helical filaments in aerial hyphae independent of ATP (Jakimowicz *et al*., 2007). These scaffolds are formed while ParB-DNA complexes are still irregular throughout the filament. This study also found that the ParA ATPase mutant forms helical filaments, but is needed for the filaments to assemble well-organized ParB complexes.

DNA Condensation

How the large, linear chromosome of *S. coelicolor* is condensed in the spore compartment still remains to be addressed. Progress has been made just this year in defining the functions of potential DNA condensation genes in the chromosome.

SMC, ScpA and ScpB homologs have been identified in *S. coelicolor*. A Δ*smc* mutant resulted in about 7% anucleate spores, compared to the 0.8% observed for wild

type (Kois *et al*., 2009). This indicates that *smc* mutants have only a slight defect in chromosome partitioning. *In vitro*, ScpA was found to interact with SMC and ScpB (Kois *et al*., 2009). These data suggests that other DNA condensation proteins must exist in *S. coelicolor* because the majority of nucleoids are still being condensed.

Spore Ploidy

Several prokaryotes were shown to produce haploid spores and therefore all were assumed to be haploid until ionizing radiation was used on the spores produced by certain *Bacillus* species. A survival curve is generated after cells are exposed to the radiation, which reveals the total surviving bacteria versus the dose of radiation. The single hit curve is exponential and indicates that with just one dose ("hit"), the cells do not survive. The multiple hit curve is sigmoidal and indicates that multiple doses ("hits") were required to kill the cells. *B. subtilis* spores were found to be inactivated by radiation in a single hit fashion, suggesting that these spores were haploid (Donnelan & Morowitz, 1957; Woese, 1958a). On the other hand, *B. megaterium*, *B. mycoides*, and *B. cereus* spores were found to have a multiple hit sigmoidal curve and were therefore considered diploid (Woese, 1958a; 1958b).

Flow cytometry, fluorescence microscopy and fluorescence *in situ* hybridization (FISH) were used to document the diploid nature of *Myxococcus xanthus* myxospores (Tzeng & Singer, 2005). *M. xanthus* is a Gram negative, rod-shaped, soil-dwelling bacterium, which upon nutrient depravation, produce fruiting bodies containing spores. Using a fluorescently labeled probe that annealed to the *oriC* region of the chromosome, it was found that vegetatively growing cells contain one or two copies of the genome, but at stationary phase, cells only contain one copy (Tzeng & Singer, 2005). The mechanism

that controls the proper ploidy of myxospores is unknown (Tzeng & Singer, 2005). Upon entry into sporulation, prespores undergo an additional round of replication. Furthermore, myxospores were found to have two genome copies; each *oriC* was localized at opposite poles of the myxospore (Tzeng & Singer, 2005). This procedure was also used to determine the location of the *ter* region of the chromosomes in the myxospores, which were found to overlap with the location of *oriC* (Tzeng & Singer, 2005). It was speculated that two copies of the chromosome per spore would enhance survival rates in the environment. Interestingly, for prokaryotes, diploidy has been only identified in spore producing bacteria, which may ensure successful germination and survival in inhospitable environments (Tzeng & Singer, 2005).

Streptomyces spore ploidy was originally investigated in 1958, when Saito & Ikeda suggested that *S. griseoflavus* spores were diploid. Their cytological analysis of *S. griseoflavus* spores indicated dumbbell DNA architecture (Saito & Ikeda, 1958). This dumbbell shape was proposed to represent two single haploid sets of genetic material in the spore, which may later condense to form a uninucleate unit (Saito & Ikeda, 1958). Based on the pattern of nucleoid staining inside the spore, Kinoshita and Itagaki (1959) reported that the majority of *Streptomyces* spores appeared to be uninucleate and, therefore, haploid. However, using the same method, some strains produced binucleate spores with a dumbbell DNA architecture, which could indicate that the spores were diploid. It was suggested that *Streptomyces* species producing uninucleate spores were the most primitive (Kinoshita & Itagaki, 1959). Because DNA methodology employed at that time was not quantitative, these results were not clear. It was possible that the nucleoid material in a spore appeared to be uninucleate, but contained two genetic units.

Microscopy was completed without present-day advancements such as fluorescent stains, confocal laser scanning microscopes, or computer software to help resolve imaging.

In 1959, Saito and Ikeda used X-ray irradiation, a more quantitative method, on *S. griseoflavus* spores and found the survival curves to have a shoulder, suggesting that there was more than one chromosome per spore. When extrapolated back to the y axis, it crossed the number two indicating that the spores were diploid. This study also examined the survival curves of spores from *S. coelicolor, S. griseus* and *S. kitasatoensis*. It was noted that *S. griseus* and *S. kitasatoensis* both had exponential survival curves, indicating that these spores were haploid. *S. coelicolor*, on the other hand, produced a sigmoidal survival curve with tailing, suggesting that the spores are diploid (Saito & Ikeda, 1959). X-ray irradiation was the best method available at that time to determine spore ploidy. This method was also used to determine that *Bacillus megaterium* spores were diploid (Woese, 1957).

Hopwood and Glauert, in 1960, stated that spores from *S. coelicolor* strain A3(2) have a one-hit survival curve, suggesting that these spores were in fact haploid. This work was cited as unpublished X-ray irradiation data from David Hopwood (Ph.D. dissertation). The field adopted the dogma that *S. coelicolor* spores were haploid and still do today, even though no concrete evidence has been provided.

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Chapter 2

Genetic interactions of *smc*, *ftsK* and *parB* genes in *Streptomyces coelicolor* and their developmental genome segregation phenotypes

ABSTRACT

The mechanisms by which chromosomes condense and segregate during developmentally-regulated cell division are of interest for *Streptomyces coelicolor*, a sporulating, filamentous bacterium with a large, linear genome. These processes coordinately occur as many septa synchronously form in syncytial aerial hyphae such that prespore compartments accurately receive chromosome copies. Our genetic approach analyzed mutants for *ftsK*, *smc,* and *parB*. DNA motor protein FtsK/SpoIIIE coordinates chromosome segregation with septum closure in rod-shaped bacteria. SMC (structural maintenance of chromosomes) participates in condensation and organization of the nucleoid. ParB/Spo0J partitions the origin of replication using a nucleoprotein complex, assembled at a centromere-like sequence. Consistent with previous work, we show that an *ftsK*-null mutant produces anucleate spores at the same frequency as the wild type strain (0.8%). We report that the *smc* and *ftsK* deletion-insertion mutants (*ftsK'* truncation allele) have developmental segregation defects (7% and 15% anucleate spores, respectively). Using these latter mutants, viable double and triple mutants were isolated in all combinations with a previously described *parB*-null mutant (12% anucleate spores).

parB and *smc* were in separate segregation pathways; the loss of both exacerbates the segregation defect (24% anucleate spores). For a triple mutant, deletion of the region encoding the FtsK motor domain and one transmembrane segment partially alleviates the segregation defect of *smc parB* (10% anucleate spores). Considerable redundancy must exist in this filamentous organism because segregation of some genomic material occurs 90% of the time during development in the absence of three functions with only a 4-fold loss of spore viability. Furthermore, we report that *scpA* and *scpAB* mutants (encoding SMC-associated proteins) have spore nucleoid organization defects. Finally, FtsK-EGFP localized as bands or foci between incipient nucleoids, while SMC-EGFP foci were not uniformly positioned along aerial hyphae, nor associated with every condensing nucleoid.

INTRODUCTION

Streptomyces coelicolor is a filamentous sporulating soil saprophyte and it is the best characterized model organism for an important group of bacteria known for their elaborate life cycle and their production of biologically active compounds. During the culmination of their life cycle, streptomycetes make chains of unicellular, metabolically-quiescent reproductive cells that subsequently separate for dispersion and long term survival in the environment (Elliot *et al*., 2008). The genome of *S. coelicolor* is a large 8.7 Mb linear molecule with a centrally located *oriC* region, ending with long terminal inverted repeats (Bentley *et al*., 2002). An aging surface-grown *Streptomyces* colony contains two basic zones of filamentous cells with different cytological properties (Flärdh, 2003). The vegetative mycelium contains branching syncytial hyphae, where the genome copies are not segregated or condensed into nucleoids and septation is infrequent and unevenly spaced. In contrast, the aerial mycelium contains mostly unbranched

syncytial aerial hyphae, and within a given hypha, uniformly-spaced septation synchronously occurs to form a chain of spores, each with a highly condensed nucleoid. Coincident with the synchronous septation, dozens of dispersed copies of the genome are accurately partitioned and condensed in the forming unicellular spores. It is conceivable that these synchronous processes coordinating the accurate separation of multiple copies of a linear genome may require a special or augmented system of segregation and condensation.

Condensation and segregation of the genome is a subject of great interest in microbial cell biology. Most of the known information has come from the study of bacterial cells that undergo binary fission (Errington *et al*., 2005; Thanbichler & Shapiro, 2008). These bacterial models typically have a single circular chromosome that is compacted into a nucleoid and genome segregation occurs simultaneously with DNA replication. In rod-shaped bacteria three well-characterized systems dovetail to ensure that chromosomes are equally and faithfully partitioned to daughter cells. FtsK (SpoIIIE), SMC (MukB) and ParAB (Soj-Spo0J) have been identified as key players. First, FtsK/SpoIIIE homologues are well studied sequence-directed DNA motor proteins that localize at the invaginating septa and clear each genome copy to the appropriate side of the septum during vegetative growth and sporulation (Barre, 2007; Bigot *et al*., 2007; Iyer *et al*., 2004; Ptacin *et al*., 2008; and references therein). The homologues typically have an N-terminal domain with membrane-spanning segments responsible for septal localization separated by a linker from a more highly conserved C-terminal motor protein domain (Massey *et al*., 2006). Second, SMC (structural maintenance of chromosomes, functional homologue MukB in *Escherichia coli*) proteins are widely distributed from

bacteria to mammals and participate in higher-levels of chromatin organization, acting either as condensins or cohesins (Graumann, 2001; Hirano, 2006; Nasmyth & Haering, 2005; Strunnikov, 2006; and references therein). DNA may be pulled into the cell by the action of these proteins from a single or limited number of sites. The molecular details of how these large hinged proteins function in association with non-SMC modulators (ScpAB/MukEF) to compact the chromosome into higher-order structures are being determined (Chen *et al*., 2008; Cui *et al*., 2008; Hirano & Hirano, 2006). Third, chromosomes from a wide range of bacteria use a partition system related to ones described for certain low-copy-number plasmids. Two *trans*-acting factors, ParA (Soj) ATPase and ParB (Spo0J), and *cis*-acting *parS* ("centromere") sites in the origin proximal region are utilized (Bignell & Thomas, 2001; Ebersbach & Gerdes, 2005; Thanbichler & Shapiro, 2008; and references therein). Spo0J (ParB) binds to the *parS* sites to form a large nucleoprotein complex that can spread beyond the origin region (Breier & Grossman, 2007). ParA is required for the proper placement of the ParB-*parS* nucleoprotein complex in the cell. For unicellular bacteria, individual inactivation of any of these three systems results in a severe segregation phenotype and combining them typically leads to synthetic lethal phenotypes.

How copies of the linear genome are synchronously partitioned during sporulation in *S. coelicolor* is beginning to be appreciated. If utilized, the physical landmarks in the apical aerial hypha, the tip (pole) and the septum at the base of the long apical cell, can be separated by 40-50 μm or more. DNA segregation occurs after active rounds of DNA replication populate the predivisional apical cell with several dozen dispersed copies of the genome (Ruban-Ośmialowska *et al*., 2006). After DNA replication ceases, septa

synchronously form over these unsegregated copies of the genome (Miguélez *et al*., 1998; Schwedock *et al*., 1997; Wildermuth & Hopwood, 1970). ParB binds to about 20 *parS* sites densely packed around *oriC* in the chromosome forming a nucleoprotein complex that helps to accurately partition the chromosome during division (Jakimowicz *et al*., 2002; Kim *et al*., 2000). ParB-EGFP localizes as regularly-spaced foci assembled along aerial hyphae before DNA segregation and septation begin, suggesting that the origin region of the chromosome is accurately positioned between where adjacent septa form (Jakimowicz *et al*., 2005). ParA assembles into helical structures along aerial hyphae to facilitate the organization of evenly-spaced ParB-*oriC* complexes (Jakimowicz *et al*, 2007). It was anticipated that the FtsK motor protein would be instrumental in ensuring that the genomic DNA trapped by invaginating septa would be properly partitioned, however, strains containing a complete deletion of *ftsK* resulted in a mild segregation phenotype (Ausmees *et al*., 2007; Wang *et al*., 2007). SffA, another FtsK/SpoIIIE-like protein, localizes to sporulation septa and may function in chromosomal DNA organization during differentiation (Ausmees *et al*., 2007). Septal localization of SffA requires a small protein called SmeA, which itself appears to have a more pleiotropic role in spore maturation. Thus, progress has been made, but there are still substantial gaps in our understanding of how the linear genome is segregated and condensed.

In this study, we evaluated the functions of *ftsK* and *smc* in *S. coelicolor* by constructing *ftsK* and *smc* deletion-insertion mutant strains. In contrast to two recent studies, the *ftsK* allele reported here results in a C-terminal truncation, removing the DNA motor domain of FtsK and a membrane–spanning segment. Each mutation studied

here was found to affect the accuracy of genome segregation into spores. We then extended our analysis using a previously published *parB* mutation. We report that all double and triple mutants are viable. Surprisingly, the majority of the spores produced by a triple mutant still receive genomic material with a modest 4-fold decrease in viability, suggesting that there are additional genes responsible for proper developmental genome segregation and condensation. In addition, we show that mutations in the genes encoding SMC-associated proteins ScpA and ScpB mainly affect the nucleoid morphology in pre-spore compartments rather than impair segregation. Finally, we determined the subcellular localization of FtsK and SMC in aerial hyphae undergoing differentiation using EGFP translational fusions.

MATERIALS AND METHODS

Bacterial strains and media

S. coelicolor A3(2) strains used in this study are listed in Table 2.1. YEME (liquid), R2YE (agar), MS (agar) and minimal medium MM (agar with 0.5% glucose, w/v) were used for growth of *S. coelicolor* at 30°C (Kieser *et al*., 2000; McCormick *et al*., 1994). Standard procedures for protoplast preparation and transformation were used (Kieser *et al*., 2000). Final concentrations of antibiotics used for *S. coelicolor* were: apramycin 25 μg ml^{-1}, neomycin 10 μg ml^{-1}, hygromycin 200 μg ml^{-1}, kanamycin 160 μg ml^{-1} and thiostrepton 50 μg ml^{-1}. Viability of wild type and certain mutant strains was determined using the average of at least two independent spore preparations (growth on MS) analyzed from duplicate serial dilutions by direct count using phase-contrast microscopy and viable count after three days incubation on MM glucose medium.

E. coli strain TG1 was used for standard plasmid manipulation (Sambrook *et al*., 1989). *E. coli* strain BW25113 containing λ RED plasmid pIJ790 (Gust *et al*., 2004) was used for *in vivo* construction of *egfp* fusions or deletion of *scpAB*. *E. coli* strain ET12567 (*dam*$^-$, *dcm*$^-$, *hsdM*$^-$) (MacNeil *et al*., 1992) was used to prepare unmodified plasmid and cosmid DNA to circumvent the restriction system of *S. coelicolor*. Final concentrations of antibiotics in LB medium used for *E. coli* were: ampicillin 100 μg ml^{-1}, carbenicillin 100 μg ml^{-1}, apramycin 100 μg ml^{-1}, chloramphenicol 25 μg ml^{-1}, kanamycin 50 μg ml^{-1}.

Plasmids and general DNA techniques

Plasmids used in this study are listed in Table 2.2. *S. coelicolor* total DNA preparations were obtained using the Wizard Genomic DNA Purification Kit (Promega). DNA restriction and modifying enzymes were used according to the manufacturer's recommendation (New England BioLabs). *Taq* (Promega) and *Pfx* (Invitrogen) DNA polymerases were used according to the manufacturer's instructions. Redirect technology (Gust *et al*., 2004) was used for λ RED-mediated recombination using mutagenic linear DNA cassettes in *E. coli*.

Isolation of *ftsK* insertion-deletion mutant strains

Insertion-deletion mutations for *ftsK* of *S. coelicolor* were constructed in *E. coli* using traditional *in vitro* recombinant DNA technology. In plasmids pJR148 and pJR152 (Table 2.2), 1.8 kb of the 3' end of the *ftsK* gene, encoding the DNA motor domain and one membrane-spanning segment, was replaced by a neomycin-resistance marker (*aphI*), inserted in either orientation. The insertion-deletion mutations in pJR148 and pJR152 were introduced into the chromosome by transformation, selecting neomycin resistance and screening for apramycin sensitivity. Two independent representative strains were

named JM148 and JM152, respectively. These strains could express a C-terminal truncated version of FtsK containing the first 203 amino acids (out of 929). In plasmid pAEB232 (Table 2.2), a 2.5 kb deletion, beginning 57 bases upstream of the translation start site and ending at the same position as above, results in the entire 5' end of the *ftsK* gene being replaced by an apramycin-resistance marker [*acc(3)IV*]. The insertion-deletion mutation in pAEB232 was introduced into the *S. coelicolor* chromosome by transformation, selecting apramycin resistance and screening for spectinomycin sensitivity. The resulting strain RMD24 should not express FtsK.

Isolation of *smc* deletion-insertion mutant strains

Insertion-deletion mutations in *smc* of *S. coelicolor* were constructed in *E. coli* using traditional *in vitro* recombinant DNA technology. Initially, we isolated a Δ*smc*::*acc(3)IV* mutant (strain HW1) to determine that the *smc* gene was dispensable (Wildschutte thesis, 2000). We isolated another *smc*-null strain, containing Δ*smc*::*hyg*, for compatibility with other mutation markers used in this study. In plasmid pHJ2 (Table 2.2), the majority of *smc* was replaced by a hygromycin-resistance gene (*hyg*). Using pHJ2, the Δ*smc::hyg* mutation was introduced into the *S. coelicolor* chromosome by transformation, selecting hygromycin resistance and screening for apramycin sensitivity. One representative strain was named HJ2.

Characterization of a *parB* mutant strain

Strain J2537 [Δ*parB*::*acc(3)IV*] strain was a gift from Keith Chater (Kim *et al*., 2000). By Southern blot hybridization analysis, we determined that the apramycin-resistance gene was in the same orientation as *parB*.

Isolation of *scpA* and *scpAB* mutants

Insertion-deletion mutations in *SCO1768*, *scpA* and *scpB* of *S. coelicolor* were constructed *in vivo* in *E. coli* using λ RED-mediated recombination and cosmid SCI51. In cosmid derivatives pRMD7, pRMD8 and pRMD9 the majority of the *SCO1768* (encoding a putative pseudouridine synthase), *scpA* or *scpAB* genes, respectively, were replaced by a cassette obtained from pIJ773 containing an apramycin-resistance gene and *oriT* flanked by FRT sites (Gust *et al*., 2004). Oligonucleotides listed in Table 3 were used as primers in PCR reactions to add homology to the ends of the apramycin-resistance gene cassette. Primers 60Sco1768 and 59Sco1768 were used for completely deleting SCO1768, oScpA60 and oScpA59 were used for deleting the middle 777 bases of the 792 base *scpA* and oScpA60 and oScpB59 were used for deleting *scpA* and the first 426 bases of the 5' end of the 672 base *scpB*. The insertion-deletion mutations in each of the cosmids were introduced into the chromosome by conjugation, selecting apramycin resistance and screening for kanamycin sensitivity. Representative strains were named RMD17 (Δ*SCO1768*::*acc(3)IV*), RMD18 (Δ*scpA*::*acc(3)IV*) and RMD19 (Δ*scpAB*::*acc(3)IV*).

Isolation of double and triple mutant strains

Double and triple mutant strains were constructed using protoplasts prepared from one mutant strain and transforming with chromosomal DNA prepared from a second mutant strain (Table 2.1). All double and triple mutant strains were readily isolated by selection using the antibiotic-resistance gene marking each insertion-deletion mutation.

Southern blot hybridization analysis

Each mutation in single, double and triple deletion-insertion mutant strains was confirmed by Southern blot hybridization analysis. Descriptions of probes used and data

obtained are located in a supplementary file. Hybridization reactions were performed at 65°C using buffer that contains 5% SDS (Virca *et al*., 1990). Nylon membranes (Hybond-N, Amersham) were used as solid support and probes were non-isotopically labeled before immunological detection (DIG DNA Labeling and Detection Kit, Boehringer Mannheim).

Construction of plasmids for genetic complementation

Genetic complementation plasmids were derived from the self transmissible, low-copy-number, bifunctional plasmid pJRM10 (Table 2.2). *ftsK*$^{+}$ is the only complete reading frame on the DNA insert of plasmid pRMD6. *smc*$^{+}$ is the only complete reading frame on the DNA insert of plasmid pHW35. The DNA insert of *scpAB* complementation plasmid pRMD11 contains 5 complete genes; *SCO1772*, *SCO1771* (hypothetical gene), *scpAB*$^{+}$, and the downstream gene *SCO1768* (putative pseudouridine synthase). The insert in pRMD11 ends 1 base downstream of *SCO1768*. These plasmids were introduced into the multiple auxotroph 2709 by transformation, selecting for thiostrepton resistance. The plasmids were mated from the 2709 derivative into the desired mutant strains selecting for thiostrepton resistance and prototrophy. Transconjugants were tested for the presence of the antibiotic-resistance genes marking the chromosomal mutations.

The strain J2540 (Δ*parB / parB*$^{+}$), and derivatives isolated here, had *parB*$^{+}$ integrated by single homologous crossover recombination using plasmid pIJ6539 (Kim *et al*., 2000).

Construction of strains expressing EGFP fusion proteins

Genes expressing EGFP fusions were constructed in one step by *in vivo* recombination. Cosmid H24*parB-egfp* (Jakimowicz *et al*., 2005) was used as the source of an *egfp acc(3)IV oriT* cassette to make *ftsK-egfp* and *smc-egfp* fusions. PCR was performed using H24*parB-egfp* as template with specific oligonucleotides which anneal to the linker sequence at the 5' end of *egfp* and downstream of the 3' end of *acc(3)IV*, and introduce 40 bases of homology to the 3' ends of *smc* or *ftsK*, as well as remove the stop codons (Table 2.3). PCR products were introduced into *E. coli* strains expressing λ Red recombination functions and containing the cognate cosmid with either *ftsK* (SC7C7) or *smc* (SC7A1). Apramycin-resistant transformants were selected creating 7C7*ftsK-egfp* and 7A1*smc-egfp*. The gene fusion junction and *egfp* sequences were verified. The proline and glycine-rich 5 amino acid-linker peptide (LPGPE) was similar to that of the H24*parB-egfp* (Jakimowicz *et al*., 2005). Fusion-containing cosmids were conjugated from *E. coli* into strain M145, selecting apramycin resistance and kanamycin sensitivity. In these transconjugants (RMD1 and RMD2), the fusion genes (*ftsK-egfp* and *smc-egfp*, respectively) are the only copy of *ftsK* or *smc*.

Microscopy

Strains were characterized by laser scanning confocal microscopy using a Leica SP2 microscope equipped with a phase-contrast 100X oil objective (1.40 NA) and 488- and 543-nm lasers. Autofluorescence seen in wide field microscopy is not an issue with confocal microscopy with appropriately adjusted excitation light. There was no background autofluorescence in the wild type strain under the conditions used. A standardized amount of spores (cfu) was inoculated on MS agar adjacent to sterile cover slips that were embedded at a 45° angle in the agar medium and incubated for the

indicated lengths of time. Coverslips were removed and cell material was fixed at room temperature for 10 min using a phosphate buffered saline (PBS) solution containing 4.375% glutaraldehyde and 0.028% paraformaldehyde. The coverslips were washed twice with PBS, allowed to air dry, and mounted in 50% glycerol containing 0.1% propidium iodide, a total nucleic acid stain. Images were processed to adjust the brightness and contrast using Adobe Photoshop and assembled in Adobe Illustrator.

Computer analysis

Predicted gene and protein products were obtained from the *S. coelicolor* genome database ScoDB (http://streptomyces.org.uk/S.coelicolor/index.html).

RESULTS

Identification of *ftsK* and *smc* homologues in *S. coelicolor*

FtsK (SpoIIIE) is a DNA motor protein that is involved in the process of chromosome partitioning during cell division or spore formation (Liu *et al*., 1998; Wu & Errington, 1994; Yu *et al*., 1998). The *ftsK* (*SCO5750*) gene is 2,790 bp and is predicted to encode a 929 amino-acid protein $FtsK_{SC}$ (Fig. 2.1A). Complementing our study, recently published articles also reported genetic analysis of *SCO5750* (Ausmess *et al*., 2007; Wang *et al*., 2007). Transmembrane prediction program analyses for $FtsK_{SC}$ are consistent with the models containing four transmembrane domains proposed for $SpoIIIE_{BS}$ (Wu & Errington, 1997) and $FtsK_{EC}$ (Dorazi & Dewar, 2000). The N-terminus is required for targeting FtsK to the division plane (Wang *et al*., 2007).

SMC proteins are extremely well conserved throughout prokaryotes, archaea and eukaryotes; they are involved in chromosome maintenance and structure (Koshland & Strunnikov, 1996; Strunnikov, 1998; Hirano, 1999). The *smc* gene of *S. coelicolor*

(*SCO5577*) is 3,561 bp in length and is predicted to encode a 1,186 amino-acid protein (Fig. 2.1B). SMC proteins consist of N- and C-terminal DNA-binding domains, connected by two coiled coil regions joined by a hinge. The highest similarities to homologues were in the N and C-terminal ATPase domains (54% and 46% identical to SMC of *B. subtilis*, respectively). The functional analog of SMC is MukB in *E. coli*.

ftsK* and *smc* are dispensable for growth and viability of *S. coelicolor

Plasmids pJR148 and pJR152 were constructed *in vitro* and contain a deletion-insertion mutation for *ftsK* marked with a neomycin-resistance gene (Δ*ftsK*::*aph*I), with *aph*I cloned in each orientation. The altered *ftsK* genes (*ftsK'*) can express a C-terminally truncated protein of 203 amino acids (FtsK'). Mainly, the motor domain was lost as the truncation is in a loop between the final two predicted transmembrane segments. Plasmid pHJ2 contains a deletion-insertion mutation for *smc* marked with a hygromycin-resistance gene (Δ*smc*::*hyg*). Using these plasmids, the mutations were introduced into the chromosome by transformation. Marker replacement strains were readily isolated indicating that these genes are not essential for growth or viability. Macroscopically, these strains looked very similar to the wild type strain. They grew well and formed a robust, gray, aerial mycelium (Fig. 2.1D). As noticed in two recently published papers, strains containing Δ*ftsK* mutations showed increased colony heterogeneity upon plating (Ausmess *et al*., 2007; Wang *et al*., 2007). The Δ*ftsK* strains reported here also had increased colony heterogeneity as did the Δ*smc* strain.

These new strains were further characterized using scanning laser confocal microscopy. The DNA segregation phenotype associated with each mutant strain was determined by staining the DNA with propidium iodide. DNA segregation did not appear

to be affected in vegetative hyphae of *ftsK* and *smc* mutants (data not shown). Figure 2.2 illustrates that spores formed in aerial hyphae in the single mutants (Fig. 2.2B-D) were similar to the wild type in shape and size (Fig. 2.2A), and had detectable defects in DNA partitioning (Table 2.4). Wild type strain produced aerial hyphae with 0.8% anucleate spores. Because of its similarity to eukaryotic condensins and cohesins, we anticipated a chromosome condensation defect in the Δ*smc* mutant, but no obvious phenotype was observed and nucleoids in mature spore compartments were no less condensed. The Δ*smc* mutant produced aerial hyphae with 7% anucleate spores, which indicated a defect in segregation. For the Δ*smc* mutant, we did not observe spores with trapped DNA inside the septa (guillotine effect), like that observed of the *mukB*-disrupted mutant in *E. coli* (Niki *et al*., 1991). Consistent with the gene product's role as a DNA motor protein, the Δ*ftsK* truncation mutants, isolated with different orientations of *aph*I, behaved similarly and produced aerial hyphae with 15-17% anucleate spores (Table 2.4). Here, in certain hyphae, we did observe DNA trapped in septa and small spore compartments with less DNA than normal (data not shown). The observed segregation defect was surprising because complete deletions of *ftsK* resulted in strains that produced spores with reduced DNA staining but no anucleate spores (Ausmess *et al*., 2007; Wang *et al*., 2007). To rule out a difference in laboratory copies of the same wild type, we isolated a strain containing a complete deletion of *ftsK* (RMD 24, Δ*ftsK::acc(3)IV*) and observed that it produced anucleate spores at a level similar to the wild type parent (Table 2.4), in agreement with the other recent studies. We concluded that expressing the truncated version FtsK'was responsible for anuclete spore production in the Δ*ftsK*::*aph*I strains (JM148 and JM152). For comparison we analyzed the previously published Δ*parB* mutant (Kim *et al*., 2000).

Analysis of Δ*parB* strain under our growth conditions indicated that it produced aerial hyphae with 12% anucleate spores (Table 2.4), the same as was originally reported. Thus, loss of *parB* or the portion of *ftsK* encoding the DNA motor domain and a membrane-spanning segment had a similar affect on the fidelity of simultaneous DNA segregation into each prespore compartment. The effect of losing *smc* was more modest and about half that observed for losing *parB* or truncating *ftsK*. Completely removing *ftsK* had no effect on anucleate spore production.

Genetic complementation experiments were completed to confirm that the segregation phenotypes observed were due to the truncation of *ftsK* or loss *smc* and not to an unlinked mutation. Wild type copies of each gene were cloned into the low-copy-number vector pJRM10 (SCP2* derivative, 1-2 copies/chromosome). The DNA inserts contained only one complete gene, *ftsK* or *smc* (Fig 2.1A-B). The phenotypes of the complemented Δ*ftsK* and Δ*smc* mutants were analyzed (Table 2.4). The Δ*ftsK*/*ftsK*$^+$ strain produced aerial hyphae with 5% anucleate spores. Thus, about two thirds of the segregation phenotype associated with the *ftsK* truncation mutation was complemented. The Δ*smc*/*smc*$^+$ strain produced aerial hyphae that produced 3% anucleate spores, indicating that 50% of the phenotype associated with the mutation was complemented. Our complementation data suggests that the defects observed in segregation resulted, at least in part, from the introduced *ftsK* and *smc* insertion-deletion mutations (see additional complementation results below). At least one promoter, active in aerial hyphae, is present on the DNA inserts used for genetic complementation. However, proper temporal and spatial expression may require that the genes be located in their native chromosomal locations.

Double mutants for Δ*smc* Δ*ftsK'*, Δ*smc* Δ*parB* and Δ*ftsK'* Δ*parB* have more severe developmental defects

For the construction of double mutants, we used Δ*ftsK'* mutant JM148 (*aph*I inserted in the same orientation as *ftsK'*) because the two mutants with either orientation of the marker had the same phenotype (Table 2.4). The double mutants for Δ*smc* Δ*ftsK'*, Δ*smc* Δ*parB* and Δ*ftsK'* Δ*parB* were constructed by transformation using DNA prepared from one mutant strain and using a second mutant as recipient (Table 2.1). The double mutant strains were readily isolated, which indicated that the three double mutants were not synthetic lethal combinations. Macroscopically, these double mutants had a wild type appearance without any obvious growth delay and formed a robust gray aerial mycelium (Fig 2.1D), although they all displayed more colony heterogeneity relative to the wild type strain. Thus, the double mutants did not even have a synthetic sick phenotype.

DNA segregation did not appear to be affected in vegetative hyphae of the double mutants (data not shown). Observations by phase-contrast microscopy revealed aerial hyphae containing spores that were more heterogeneous in size and shape compared to that of the single mutants or wild type. In addition, Fig. 2.2E-G illustrates that spore chains in the double mutants had defects in developmental DNA partitioning and the segregation defect in each strain was quantified (Table 2.4). The Δ*smc* Δ*ftsK'* mutant produced aerial hyphae containing 13% anucleate spores, similar to those of the Δ*ftsK'* strain (Fig. 2.2E). Therefore, additive effects of the mutations were not seen when combining the loss of a condensation gene (7%) with the segregation gene (15%). Interestingly, the Δ*ftsK'* Δ*parB* mutant also was only 13% anucleate, which again indicated that this strain did not have segregation defects additive of those observed for

the Δ*ftsK'* (15%) and Δ*parB* (13%) single mutant strains (Fig 2.2G and Table 2.4). It seems that without the function of the two DNA partitioning and segregation genes, *S. coelicolor* still can disseminate its DNA just as well as the most defective single mutant. In contrast, the Δ*smc* Δ*parB* mutant produced aerial hyphae containing 24% anucleate spores (Fig. 2.2F), slightly more than the additive effect (19%) of each individual gene loss (Table 2.4). Commensurate with the rise in the segregation defect, the Δ*smc* Δ*parB* mutant had a more defective developmental phenotype than the other double mutants. Aerial hyphae of the Δ*smc* Δ*parB* mutant contained more spore compartments with altered shapes and sizes. This observation, along with the single mutant data, suggested that there was a phenotypic effect when deleting both a partitioning and condensation gene in *S. coelicolor*, indicating genetic overlap or interaction.

In order to verify that the phenotypes in the double mutants resulted from the introduced mutations, genetic complementation analyses were completed with the low-copy-number plasmids bearing *smc* or *ftsK* or an integrated copy of *parB* (Table 2.4). Complementation data for the double mutant strains suggests that the phenotypes were linked to the mutations introduced by transformation into the Δ*smc* mutant. The Δ*smc* Δ*ftsK'*/*ftsK*$^+$ strain produced aerial hyphae containing spores that were 7% anucleate, which indicates the *ftsK* truncation mutation was completely complemented. The Δ*smc* Δ*parB*/*parB*$^+$ strain produced aerial hyphae that contained 6% anucleate spore, also indicating complete complementation. Finally, the Δ*ftsK'* Δ*parB*/*ftsK*$^+$ strain produced aerial hyphae with 16% anucleate spores, which is similar to the 13% anucleate spores in aerial hyphae of the Δ*parB* strain. Complementation data suggests that the introduced

second mutation was responsible for the observed phenotypes when compared to the single mutant strains.

An Δ*smc* Δ*parB* Δ*ftsK'* triple mutant is viable and does not have as severe of a developmental segregation defect as a Δ*smc* Δ*parB* double mutant

The triple mutant Δ*smc* Δ*parB* Δ*ftsK'* was easily isolated by transformation with DNA prepared from a Δ*parB* strain and using the Δ*smc* Δ*ftsK'* strain as recipient. Macroscopically, the triple mutant showed no obvious growth delay and produced a robust gray aerial mycelium (Fig 2.1D), but an elevation in colony heterogeneity was evident. Mutations in the three genes did not result in a synthetic lethal or synthetic sick phenotype.

Using phase-contrast microscopy, the spores of the Δ*smc* Δ*parB* Δ*ftsK'* strain appeared heterogeneous in shape and size, most like those of the Δ*smc* Δ*parB* strain (Fig 2.2H). Segregation did not appear to be affected in vegetative hyphae of the triple mutant (data not shown). Surprisingly, determination of the segregation phenotype for the triple mutant indicated that only 10% of the spores were anucleate (Table 2.4). The number of anucleate spores in aerial hyphae for the Δ*smc* Δ*parB* Δ*ftsK',* strain was not the total sum of each single mutant combined (34%). It was not even as poor as the most severe phenotype of the double mutant strains (Δ*smc* Δ*parB*, 24%). Without all three of these genes, 90% of the spores are still receiving some genomic material. This observation suggests that there must be considerable redundancy and another unidentified system for developmental DNA segregation must function in this filamentous organism.

We performed genetic complementation analyses with the low-copy-number plasmids bearing *smc* or *ftsK* (Table 2.4). In particular, the complementation strain with

the genotype Δ*smc* Δ*parB* Δ*ftsK'*/*ftsK*$^{+}$ produced aerial hyphae with an increase in the number of anucleate spores to 27%, which was similar to the Δ*smc* Δ*parB* phenotype of 24% (Table 2.4). This verified that the introduced Δ*ftsK'* truncation mutation was responsible for partially alleviating the segregation defects in the Δ*smc* Δ*parB* strain. Therefore, introducing a functional copy of *ftsK* back into the triple mutant made the genome segregation phenotype worse, suggesting that these genes interact genetically.

The fact that only 10% of a triple mutant spores were anucleate prompted us to test if the viability of the spores was more adversely affected and the percent anucleate values were misleading. The viability of spores plated on minimal medium supplemented with glucose was compared for the most pertinent strains. Wild type strain produced spore compartments that were 49% viable. Although the Δ*ftsK'* truncation and Δ*smc* Δ*parB* mutants have a significant number of anucleate spores (15% and 24%, respectively), surprisingly the viability of those produced was equal to or slightly higher than the wild type, respectively. In contrast, spores of the triple mutant were 12.5% viable, one forth that of wild type. Although 90% of the spores in the triple mutant receive some genetic material, they are four-fold less fit than wild type.

FtsK-EGFP localizes between nucleoids of aerial hyphae

ftsK was replaced by *ftsK-egfp* in the chromosome of the wild type strain. This strain (RMD1) produced aerial hyphae containing spores with normal size and shape, as judged by phase-contrast microscopy. DNA segregation defects were not observed, as judged by fluorescence microscopy (data not shown). We concluded that the FtsK-EGFP fusion protein was functional.

Localization of FtsK-EGFP was analyzed at several time points during the life cycle and FtsK-EGFP foci were apparent at both early vegetative and early aerial hyphae stages of growth. FtsK-EGFP was localized as distinct foci at several places within young germ tubes growing out of spores (Fig 2.3A). Distinct gaps in the propidium iodide staining were repeatedly observed and FtsK-EGFP foci coincided with those gaps in staining. This result suggested that DNA segregation could be occurring during outgrowth of newly germinated spores and there was a role for FtsK in this process. However, gaps in the propidium iodide staining were observed for *ftsK* mutants as well, indicating the gaps in staining were not solely caused by the action of FtsK (data not shown). Although we attempted to stain membranes and cross-walls with unsatisfactory results, we assume that at least some gaps in DNA result from vegetative cross-walls. In addition, FtsK-EGFP localized as foci or bands in reproductive aerial hyphae prior to the formation of sporulation septa (Fig 2.3B). The ladder-like pattern in the aerial hyphae was similar to what was observed for FtsZ localization (Grantcharova *et al*., 2005; Schwedock *et al*., 1997). This data indicated that FtsK localized to evenly-spaced positions expected if it were to have a role in correctly positioning the nucleoids into prespore compartments before the DNA becomes trapped by the invaginating septa. Similar FtsK localization during synchronous septation in aerial hyphae was independently reported (Ausmess *et al*., 2007; Wang *et al*., 2007). In addition, we also observed potential helical structures that preceded the appearance of the evenly-spaced ladder-like arrays as reported by Wang *et al*. (2007).

SMC-EGFP is localized in a punctuate pattern in aerial hyphae

smc was replaced by *smc-egfp* in the chromosome of the wild type strain. This strain (RMD2) produced aerial hyphae containing spores with normal size and shape, as judged by phase-contrast microscopy. DNA segregation defects were not observed, as judged by fluorescence microscopy (data not shown). We concluded that the SMC-EGFP fusion protein was functional.

We anticipated that SMC might bind to every chromosome, helping to condense the DNA into nucleoids during genome segregation into prespore compartments. However, SMC-EGFP foci were not uniformly positioned and seemed to localize in a punctate pattern only in pre-divisional aerial hyphae. The foci were not observed to be associated with every nucleoid, nor with any particular region of the aerial filament (Fig. 2.3C). SMC-EGFP signal was not observed at any other time in the lifecycle. Similar observations were made using immunofluorescence microscopy and an SMC-specific antibody (Kois *et al*., 2009), indicating that the SMC-EGFP fusion did reflect the behavior of native SMC.

scpA and _scpB_ mutants cause DNA condensation defects

SMC functions in association with non-SMC proteins called ScpA and ScpB (segregation and condensation protein) (Mascarenhas *et al*., 2002; Soppa *et al*., 2002). These proteins (MukEF) may regulate SMC (MukB) association with DNA (Mascarenhas *et al*., 2005; Petrushenko *et al*., 2006). *scpA* (*SCO1770*) is 795 bp and is predicted to encode a 264 amino-acid protein and *scpB* (*SCO1769*) is 672 bp and is predicted to encode a 223 amino-acid protein (Fig. 2.1C). ScpA and ScpB are approximately 27% and 32% identical, respectively, to the *B. subtilis* homologues. The genes encoding ScpA and ScpB are adjacent and are likely to be co-transcribed with

SCO1768, predicted to encode a pseudouridine synthase (Fig. 2.1C). Plasmids were constructed *in vitro* containing deletion-insertion mutations for *SCO1768, scpA* and *scpAB* marked with an apramycin-resistance cassette (Table 2.2). These mutations were introduced individually into the chromosome by conjugation and marker replacement strains were readily isolated (Table 2.1), indicating that these genes are not essential for growth or viability. These strains produced the normal grey, aerial mycelium characteristic of the wild type strain (data not shown).

Deletion of *rluD*, coding for one of the many pseudouridine synthases in *E. coli*, causes a growth rate defect (Gutgsell *et al*., 2001). Because *scpB* and *SCO1768* overlap by 1 base and mutations in *scpA* or *scpB* were likely to have polar effects on the expression of *SCO1768*, we isolated a *SCO1768* mutant to determine its phenotype in *S. coelicolor*. The introduced mutation for *SCO1768* did not have an obvious effect on the growth rate, nor did it have a DNA segregation or condensation phenotype (data not shown). We conclude that the phenotypic effects observed for the Δ*scpA* or Δ*scpAB* mutants resulted from the deletion of those genes.

We anticipated that the mutants for *scpA* or *scpAB* might have a segregation phenotype similar to that observed for *smc*. However, the observed frequency of anucleate spores was 1% (Table 2.5), insignificantly higher than wild type (0.8%) and less than *smc* deletion-insertion mutant (7%, Table 2.4). Instead, a substantial fraction of nucleoids of the Δ*scpA* and Δ*scpAB* strains had an altered morphology in sporulating aerial hyphae (Fig 2.4). Certain nucleoids did not follow the contours of the spore compartment and contain two lobes of DNA, a phenotype we have called “bilobed”. In late aerial filaments, 15% spores in the Δ*scpA* strain and 26% spores in the Δ*scpAB* strain

were bilobed (Table 2.5). In contrast, bilobed spores were not observed in the wild type strain. Although the isolated deletion of *scpA* might be polar on the expression of *scpB*, deleting both genes resulted in a higher frequency of bilobed spores indicating that some *scpB* must be expressed in the *scpA* mutant. Genetic complementation experiments indicated that the observed nucleoid morphology defect was the result of the introduced deletion-insertion mutation for *scpA* or both *scpAB*. Introduction of pRMD11, containing *scpAB*$^{+}$ and *SCO1768*, substantially reduced the occurrence of bilobed spores in the mutants (Table 2.5).

Because ScpA and ScpB are thought to regulate the interaction of SMC with DNA, we were interested in learning the phenotypes obtained when combining *scpA* or *scpAB* mutations with *smc* (Table 2.1). The data suggests that deleting *smc* in the *scpA* or *scpAB* mutant backgrounds combines their effects. While bilobed spores were not observed for the Δ*smc* mutant, aerial hyphae of Δ*smc* Δ*scpA* double mutant produced spores with 14% bilobed nucleoid morphology and 4% anucleate (Table 2.5). Aerial hyphae of the Δ*smc* Δ*scpAB* triple mutant produced spores with 17% biolobed nucleoid morphology and 3% anucleate. Approximately a similar number of spore compartments were affected in the triple mutant compared to the *scpAB* double mutant where the percentage of bilobed spores decreased and the percentage of anucleate spores increased. The data are consistent with an interpretation that ScpA and/or ScpB have functions in addition to regulating SMC activity.

DISCUSSION

smc and chromosome partitioning.

In this study we described the isolation of an *smc* mutant. The main defect observed was in segregation not in DNA condensation. The magnitude of the segregation defect in the *smc* mutant was modest resulting in 7% anucleate prespore compartments, less than 10-fold more than that of the wild type parent. The assembly of SMC condensation complexes presumably functions to compact and help pull the linear genome into the space available in prespore compartments. Consistent with the expected function and genetic defect, SMC-EGFP foci form in irregular patterns along aerial hyphae at a stage prior to when synchronous septation occurred and nucleoids were condensed. These results may suggest that nucleoid condensation in adjacent compartments of aerial hyphae is non-synchronous. Alternatively, SMC-EGFP may not aggregate to discrete foci that can be visualized easily. SMC-EGFP foci were not observed in vegetative hyphae or in prespore compartments (i.e., post septation). This could indicate that expression is developmentally regulated and SMC is present in a low concentration in vegetative hyphae, where DNA is not condensed, or early aerial hyphae, before DNA segregation occurs. Further regulation may be at the level of activity. SMC may only transiently and dynamically localize at points in the lifecycle, such as when particular DNA sequences must be condensed in the maturing spore compartments. Similar results for the segregation phenotype of an *smc* mutant and SMC localization of have been obtained independently (Kois *et al*., 2009).

***ftsK* and chromosome partitioning.**

In this study, we also described the isolation of mutants with different deletion-insertion mutations for *ftsK*. Consistent with two recent studies (Ausmess *et al*., 2007; Wang *et al*., 2007), genome segregation during spore formation was not as appreciably

affected as anticipated with an *ftsK* null mutation (Δ*ftsK*::*acc(3)IV*). Spores with reduced DNA content were readily apparent but anucleate spores were observed at the same level as the wild type. Other *ftsK* mutants described here (Δ*ftsK*::*aphI*) harbor a mutant allele (*ftsK'*) lacking the region encoding the DNA motor domain, but still have the potential to produce a truncated protein of 203 native amino acids containing several N-terminal membrane-spanning segments (3 out of 4). In contrast, the truncation of *ftsK* resulted in a more deleterious segregation phenotype producing 15% anucleate spores, ~20-fold higher than that of wild type. Interestingly, despite the obvious increase in missegregation, the spores produced did not suffer a dramatic loss of fitness and were as viable as the wild type when germination and growth was tested on minimal medium with glucose (46% vs. 49%, respectively).

Everything else in growth and development appeared to be normal except segregation in the mutant with a truncated *ftsK* gene: aerial mycelium formation, septation, septum spacing, spore maturation (compartment rounding up) and spore pigment production. In addition, FtsZ-EGFP localized correctly indicating that the C-terminal motor domain is not required for, and the N-terminal fragment did not interfere with, Z ring formation (data not shown). Thus, FtsK' does not appear to be pleiotropically poisoning a central process such as the general secretion/membrane insertion pathway. A segregation phenotype is what would be predicted based on previous work in other organisms and that is in fact the phenotype observed. Because the phenotype of *ftsK'* is different from the loss of the entire gene, we think that the results are consistent with the interpretation that the FtsK' fragment (truncated cleanly between the third and fourth predicted transmembrane helices) associates with the components

FtsK may normally interact. We predict that FtsK' is where it is supposed to be, but it is unable to transport DNA. We conclude that the FtsK motor domain is important for proper genome segregation during prespore formation and that the N-terminal 203 amino acid FtsK' must interact with some component during prespore formation. The expression of FtsK' did not result in a dominant-negative phenotype as the genome segregation defect can be complemented by expression of *ftsK*$^{+}$ from a low-copy-number plasmid (Table 2.4). In speculation, this may indicate that the first three transmembrane segments of the FtsK/SpoIIIE family are important for the majority of normal function and the final transmembrane segment is responsible for positioning the DNA motor domain on the correct side of the membrane.

Consistent with the expected function of moving DNA trapped by invaginating septa, FtsK-EGFP foci and bands were observed to form in regular ladder-like patterns along aerial hyphae at a stage when synchronous septation occurred and the nucleoids would become condensed. Our data supports similar observations from two other recent studies (Ausmess *et al*., 2007; Wang *et al*., 2007). In addition, FtsK-EGFP foci were observed in germ tubes during spore outgrowth suggesting that FtsK might function in the vegetative hyphae, presumably moving DNA trapped when forming the widely-spaced vegetative cross walls.

Genetic interactions between segregation and condensation genes.

We combined our mutations for *smc* and truncated *ftsK* (*ftsK'*) with each other and that of *parB*. ParB is responsible for organizing the origin proximal region of the linear chromosomes into a nucleoprotein complexes that are synchronously positioned at regularly-spaced distances to help ensure that copies of the genome are captured within

each prespore compartment (Jakimowicz *et al*., 2005; Jakimowicz *et al*., 2007). A *parB* mutant produces 13% anucleate spores, ~20-fold higher than the wild type (Table 2.4 and (Kim *et al*., 2000)). Combining *ftsK'* with *smc* or *parB* mutations did not give an additive effect and the production of anucleate spores was approximately 13% for both double mutants, similar to the worst single mutant. In contrast, *smc* and *parB* are in two pathways as combining the two mutations led to 24% anucleate spores, approximately the sum of the phenotypes for each single mutant. Despite the obvious increase in missegregation, the spores produced by an *smc parB* mutant did not suffer a dramatic loss of fitness and were slightly more viable than the wild type when germination and growth was tested on minimal medium with glucose.

The most interesting result was observed when we isolated an *smc parB ftsK'* triple mutant. We anticipated that removing $ftsK^+$ would exacerbate the segregation phenotype. Instead, introducing the *ftsK'* mutation into *smc parB* background improved the ability to segregate genomic material into prespore compartments (24% anucleate for the double mutant vs. 10% for the triple mutant). We confirmed the alteration in the segregation phenotype resulted from the introduced *ftsK'* mutation. Genetic complementation of *ftsK'* (*smc parB ftsK'*/$ftsK^+$) resulted in a phenotype similar to the *smc parB* double mutant (27% anucleate spores). Our data indicate that there must be considerable redundancy and more genes that can provide backup function in segregation when *smc*, *parB* and *ftsK* are inactive or we are left to conclude that unassisted post-replication diffusion of nucleoids accounts for the even distribution of genomes during development. In the absence of the three well-characterized genes involved in bacterial genome segregation, normally resulting in lethal or synthetic lethal phenotypes when

combined in rod-shaped bacteria, an impressive 90% of the spore compartments receive at least some genomic material. A portion of those genomes in spore compartments must be complete or nearly complete copies as the triple mutant spores are approximately 25% as viable as those produced by the wild type parent when tested on minimal medium with glucose. Certainly some genomes must be guillotined by the septa and account for the loss of viability.

When normal genome segregation is disturbed by the loss of ParB and SMC, the FtsK DNA motor domain may clear trapped genomes to the "wrong" side of invaginating septa too quickly resulting in 24% anucleate compartments. When FtsK' is expressed a less efficient backup system(s) may function in the *smc parB* mutant. The less efficient redundant system is able to eventually segregate genomes into a larger proportion of prespore compartments when the strong FtsK motor is not rapidly clearing DNA from invaginating septa. A better understanding of segregation will require additional analysis. One obvious candidate might be the recently described FtsK-like protein SffA, although the functions of SffA and FtsK did not appear to overlap (Ausmees *et al*., 2007). Due to the considerable redundancy demonstrated here, the contributions of SffA may not be apparent until they are tested in *smc parB* or *smc parB ftsK'* mutants. Related to the subtle phenotype of an *sffA* mutant (Ausmees *et al*., 2007), infrequent spores with double the DNA intensity were not observed adjacent to anucleate spores in any of the mutants described here. Likewise, another *ftsK*-like gene (*SCO4508*) could be a candidate and was tested for a role in segregation (Wang *et al*., 2007) but its function may not be apparent until combined with the other genes tested here. Due to the lack of compatible markers, we have yet to test the effect of *ftsK*-null allele when combined with

smc parB. Finally, we anticipate that additional components influencing segregation might be cytoskeletal proteins MreB or Mbl (Defeu & Graumann 2003; Gitai *et al*., 2005; Srivastava *et al*., 2007) or genes under the regulation of SsgC (Noens *et al*., 2005).

Genetic instability of the linear genome.

A recent study with an *ftsK*-null mutant reported that portions of the right end of the chromosome were frequently lost in some colonies during routine propagation of the mutant strain (Wang *et al*., 2007). The interpretation of that study was that loss of FtsK resulted in an increase in genome instability. We and Ausmees *et al*. (Ausmees *et al*., 2007) also observed an increase of colony heterogeneity with *ftsK* mutants. However, we note that an increase in genome instability resulted in most of our mutants. We believe that increased genome instability is not specific for the loss of FtsK sweeping the arms of the linear chromosome from being strangled and lost, but is a property of DNA segregation mutants in general. To test if the segregation mutants in this study suffered unanticipated genetic loss, we subjected all of our strains to Southern blot hybridization analyses using the inserts of two non-overlapping cosmids as probes to see if there were any observable differences in *cmlR* (*SCO7526*) region near the right end of the chromosome. The strains from this study did not have an observable deletion or rearrangement (data not shown).

Nucleoid morphology defects in a *scpAB*-null mutant.

We anticipated that mutants for genes encoding non-SMC segregation and condensation proteins, ScpA and ScpB, would have a phenotype similar to the *smc* mutant, but perhaps more modest (Mascarenhas *et al*., 2002). These proteins were thought to interact with SMC to influence the interaction of SMC with DNA and are

required to form higher order structures involved in genome condensation. However, the phenotypes of the *smc* mutant and *scpA* or *scpAB* mutants are distinct. The loss of *scpA* or *scpAB* mainly affected the morphology of the condensed nucleoid and did not give rise to a significant segregation defect. Isolation of *smc scpA* and *smc scpAB* mutants indicated that the phenotypes of the double and triple mutant were additive. These mutants have both a segregation phenotype (*smc*) and a nucleoid morphology phenotype (*scpAB*). We speculate that in addition to the normal interaction with SMC, the nucleoid morphology perturbation might be the result of ScpA and ScpB interacting with additional proteins required for and regulating processes associated with condensation and genome organization. The SMC-ScpA-ScpB complex was shown to have a role in DNA repair and gene regulation in *B. subtilis* (Dervyn *et al.*, 2004). Those authors proposed that these roles are evolutionarily conserved and our data may agree.

Finally, the bilobed morphology of the nucleoids observed for the *scpA* and *scpAB* mutants could offer insight into the way the genome is stored in the quiescent spore. The genome in streptomycetes is a linear DNA molecule with terminal inverted repeats (TIRs). In vegetative hyphae, evidence suggests that the TIRs are held in association near each other (Yang & Losick, 2001) and perhaps the ends are also normally in association in the spore. In the *scpAB* mutants, the TIRs may no longer be closely associated revealing two independently folding domains of the nucleoid. Alternatively, a DNA replication checkpoint may have failed and the mutant prespore compartments might undergo an additional round of DNA replication. Other possibilities exist and we will be exploring the problem in future studies.

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Table 2.1

S. coelicolor A3(2) strains used in this study.

Strains	Genotype[a]	Isolation[b]	Reference
2709	*proA1 hisA1 argA1 cysD18 uraA1 strA1*		Kieser *et al*.2000
HJ2	Δ*smc::hyg*	M145 x pHW30	This paper
J2537	Δ*parB::acc(3)IV*		Kim *et al*.2002
J2540	Δ*parB::acc(3)IV / parB+*	J2537::pIJ6539	Kim *et al*.2002
JM148	Δ*ftsK::aphI* (*aphI* same orientation as *ftsK*)	M145 x pJM148	This paper
JM152	Δ*ftsK::aphI* (*aphI* opposite orientation as *ftsK*)	M145 x pJM152	This paper
M145	prototroph $SCP1^-$ $SCP2^-$		Kieser *et al*.2000
RMD1	*ftsK-egfp acc(3)IV*	M145x7C7*ftsK-egfp*	This paper
RMD2	*smc-egfp acc(3)IV*	M145x7A1*smc-egfp*	This paper
RMD3	Δ*smc::hyg* Δ*ftsK::aphI*	HJ2 x JM148	This paper
RMD4	Δ*smc::hyg* Δ*parB::acc(3)IV*	HJ2 x J2537	This paper
RMD5	Δ*parB::acc(3)IV* Δ*ftsK::aphI*	J2537 x JM148	This paper
RMD6	Δ*smc:: hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI*	RMD3 x J2537	This paper
RMD7	Δ*smc::hyg* / smc^+	HJ2 / pHW35	This paper
RMD8	Δ*ftsK::aphI* / $ftsK^+$	JM148 / pRMD6	This paper
RMD9	Δ*smc::hyg* Δ*ftsK::aphI* / $ftsK^+$	RMD3 / pRMD6	This paper
RMD10	Δ*smc::hyg* Δ*ftsK::aphI* / smc^+	RMD3 / pHW35	This paper
RMD11	Δ*smc::hyg* Δ*parB::acc(3)IV* / smc^+	RMD4 / pHW35	This paper
RMD12	Δ*smc::hyg* Δ*parB::acc(3)IV* / $parB^+$	J2540 x HJ2	This paper
RMD13	Δ*parB::acc(3)IV* Δ*ftsK::aphI* / $ftsK^+$	RMD5 / pRMD6	This paper
RMD14	Δ*parB::acc(3)IV* Δ*ftsK::aphI* / $parB^+$	J2540 x RMD5	This paper
RMD15	Δ*smc::hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI* / $ftsK^+$	RMD6 / pRMD6	This paper
RMD16	Δ*smc::hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI* / smc^+	RMD6 / pHW35	This paper
RMD17	Δ*sco1768::acc(3)IV*	M145 x pRMD7	This paper
RMD18	Δ*scpA::acc(3)IV*	M145 x pRMD8	This paper
RMD19	Δ*scpAB::acc(3)IV*	M145 x pRMD9	This paper
RMD20	Δ*smc::hyg* Δ*scpA::acc(3)IV*	M145 x RMD18	This paper
RMD21	Δ*smc::hyg* Δ*scpAB::acc(3)IV*	M145 x RMD19	This paper

Table 2.1 (continued)

Strains	Genotype[a]	Isolation[b]	Reference
RMD22	Δ*scpA::acc(3)IV / scpA*$^+$	RMD18 x pRMD10	This paper
RMD23	Δ*scpAB::acc(3)IV / scpAB*$^+$	RMD19 x pRMD10	This paper
RMD24	Δ*ftsK::acc(3)IV* (deletion begins upstream of 5')	M145 x pAEB232	This paper

a – All mutants are derivatives of prototrophic strain M145
b – Strains constructed by protoplast transformation: recipient strain first and source of donor DNA second (i.e., HJ2 x JM148, indicates that HJ2 was transformed with DNA prepared from JM148) or recipient strain and mutagenic plasmid (M145 x pHJ2).

Table 2.2

Plasmids and cosmids used in this study.

Plasmid	Description	Reference
SC7C7	cosmid source of *smc*	Redenbach *et al.*1996
SC7A1	cosmid source of *ftsK*	Redenbach *et al.*1996
SCI51	cosmid source of *scpAB*	Redenbach *et al.*1996
H24*parB-egfp*	*egfp* inserted in-frame at 3' of *parB* in cosmid SCH24, *acc(3)IV aphII*	Jakimowicz *et al.*2005
7A1*smc-egfp*	*egfp i*nserted in-frame at 3' of *smc i*n cosmid SC7A1, *acc(3)IV aphII*	This study
7C7*ftsK-egfp*	*egfp i*nserted in-frame at 3' of *ftsK i*n cosmid SC7C7, *acc(3)IV aphII*	This study
pAEB228	4.2 kb *Pvu*II frgament from SC7C7 containing *ftsK* cloned into *Eco*RV-digested pBluescript (*ftsK* same orientation as *lacZ'*)	This study
pAEB232	2.5 kb *Mlu*I-*Sph*I deletion of pJR143 (Δ*ftsK::acc(3)IV*, same orientation as *ftsK*) *aadA*	This study
pAH137	source of neomycin-resistance gene *aph*I	A. Hausler,unpublished
pBluescript II SK (+)	standard cloning vector	Stratagene
pIJ773	source of *acc(3)IV* for *in vivo* recombination	Gust *et al.*2004
pIJ6539	5.3 kb *Eco*RI fragment containing *parB, tsr (parB* complementation plasmid)	Kim *et al.*2000
pKC1053	source of hygromycin-resistance gene, *hyg*	Kuhstoss & Rao,1991
pHJ2	3.2 kb *Sal*I-*Pst*I deletion of pHW25 (Δ*smc*::*hyg*, same orientation as *smc*), *acc(3)IV*	This paper
pHW24	5.4 kb *Xho*I fragment from SC7A1 cloned into pBluescript (*smc* opposite orientation of *lacZ'*)	This paper
pHW25	5.4 kb *Xho*I fragment from SC7A1 cloned into pBluescript (*smc* same orientation of *lacZ'*)	This paper
pHW35	24 kb *Bam*HI-*Eco*RI fragment from pJRM10 cloned into pHW24 (*smc* complementation plasmid)	This paper
pJR143	6.1 kb *Bam*HI-*Pst*I fragment of SC7C7 containing *ftsK* cloned into pOJ260 (*ftsK* same orientation of *lacZ'*), *acc(3)IV*	This paper
pJR148	1.8 kb *Sph*I fragment deletion of pJR143 (Δ*ftsK*::*aphI*, same orientation as *ftsK'*), *acc(3)IV*	This paper
pJR152	1.8 kb *Sph*I fragment deletion of pJR143 (Δ*ftsK*::*aphI*, opposite orientation as *ftsK'*), *acc(3)IV*	This paper
pJRM10	bifunctional cloning vector, low-copy-number in *S. coelicolor*, *bla tsr*	McCormick & Losick 1996
pOJ260	pUC-like plasmid containing *oriT* and *acc(3)IV* in place of *bla*	Bierman *et al.*1993
pOJ427	source of apramycin-resistance gene *acc(3)IV* for *in vitro* recombination	B. Schoner,unpublished
pRMD6	24 kb *Eco*RI-*Hin*dIII fragment from pJRM10 cloned into pAEB228 (*ftsK* complementation plasmid)	This paper
pRMD7	cosmid SCI51 containing Δ*SCO1768*::*acc(3)IV,* by *in vivo* recombination, *aphII*	This paper

Table 2.2 (continued)

Plasmid	Description	Reference
pRMD8	cosmid SCI51 containing Δ*scpA*:: *acc(3)IV,* by *in vivo* recombination, *aphII*	This paper
pRMD9	cosmid SCI51 containing Δ*scpAB*:: *acc(3)IV,* by *in vivo* recombination, *aphII*	This paper
pRMD10	4.8 kb *Xba*I fragment from SCI51 containing *scpAB* cloned into pBluescript (*scpAB* opposite orientation as *lacZ'*)	This paper
pRMD11	24 kb *Hin*dIII-*Spe*I fragment from pJRM10 cloned into pRMD10 (*scpAB* complementation plasmid)	This paper

TABLE 2.3

Oligonucleotides used in this study.

Oligonucleotide	Sequence[a]	Application
oSMCgfpF	CGGTGTGTCGAAGGTCATCAGCCAGCGGTTGCGTCAGCCCCTGCC GGGCCCGGAGCTG	Construction of *smc-egfp*
oSMCgfpR	GAAGTGGTTATGTGTTCAAGTCTTGAAGAACGAGGGTTCACATAT GTAGGCTGGAGCTGCTTC	Construction of *smc-egfp*
oKgfpF	GGACGGCGTGCTCGCGGTGATCCGTGGGGAGTCTGAAGGGCTGC CGGGCCCGGAGCTG	Construction of *ftsK-egfp*
oKgfpR	CGGCGGATCGTCGGCGGAACCCTTCTCCTACCCGCCCCTACATAT GTAGGCTGGAGCTGCTTC	Construction of *ftsK-egfp*
60Sco1768	CCGGGGACGCAGACGACAAGACGGAATTTTG**ATG**CGAAGCATTC CG GGGATCCGTCGACC	Construction of Δ*SCO1768::acc(3)IV*
59Sco1768	TCGTCGGGGCAGGGGTTGCCCAGAGGCCGGT**CTA**GAGGTCTGTA GGCTGGAGCTGCTTC	Construction of Δ*SCO1768::acc(3)IV*
oScpA60	CTCCGACGACCCGGACGACGGTGTCTTCAAG**GTG**CGGCTCATTC CGGGGATCCGTCGACC	Construction of Δ*scpA*::*acc(3)IV*
oScpA59	GTGGCCTCCTCGGCCTCCGTGATCCGCTCAC**TCA**CGCCTTTGTAG GCTGGAGCTGCTTC	Construction of Δ*scpA::acc(3)IV*
oScpB59	TGCCCGCCTCCTCGACCAGACCCCGCTGCAGGAGGGTGCGTGTA GGCTGGAGCTGCTTC	Construction of Δ*scpAB::acc(3)IV*

[a]start codons and reverse complement of stop codons are bolded.

TABLE 2.4

Quantitative analysis of genome segregation defects during spore formation.

Strain	Anucleate Spores[a]	Total Spores
wild type	0.8%	2233
Δ*ftsK:: acc(3)IV* (null)	0.8%	1876
Δ*ftsK::aphI* (*aphI* in same orientation as *ftsK'*)[b]	15%	3469
Δ*ftsK::aphI* (*aphI* in opposite orientation as *ftsK'*)	17%	1463
Δ*smc::hyg*	7%	4122
Δ*parB::acc(3)IV*	12%	1783
Δ*smc::hyg* Δ*ftsK::aphI*	13%	2389
Δ*smc::hyg* Δ*parB::acc(3)IV*	24%	3663
Δ*parB::acc(3)IV* Δ*ftsK::aphI*	13%	3230
Δ*smc::hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI*	10%	1503
Δ*smc::hyg* / *smc*$^+$	3%	2033
Δ*ftsK::aphI* / *ftsK*$^+$	5%	2180
Δ*smc::hyg* Δ*ftsK::aphI* / *ftsK*$^+$	7%	2008
Δ*smc::hyg* Δ*ftsK::aphI* / *smc*$^+$	11%	1667
Δ*smc::hyg* Δ*parB::acc(3)IV* / *smc*$^+$	14%	1809
Δ*smc::hyg* Δ*parB::acc(3)IV* / *parB*$^+$	6%	1807
Δ*parB::acc(3)IV* Δ*ftsK::aphI* / *ftsK*$^+$	16%	2009
Δ*parB::acc(3)IV* Δ*ftsK::aphI* / *parB*$^+$	18%	2125
Δ*smc::hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI* / *ftsK*$^+$	27%	2075
Δ*smc::hyg* Δ*parB::acc(3)IV* Δ*ftsK::aphI* / *smc*$^+$	11%	1712

[a] One hundred random spore chains were quantified and the percentage of anucleate spores were determined. All spores were considered regardless of the compartment size. [b] Double and triple mutants contained this allele of *ftsK'*.

TABLE 2.5

Quantitative analysis of *scp* mutant genome segregation and nucleoid morphology phenotypes during spore formation.

Strain	Anucleate Spores[a]	Bilobed Spores[a]	Total Spores
wild type	0.8%	0%	2233
Δ*scpA::acc(3)IV*	1%	15%	1797
Δ*scpAB::acc(3)IV*	1%	26%	1787
Δ*scpA::acc(3)IV / scpAB*$^{+}$	1%	2%	1790
Δ*scpAB::acc(3)IV / scpAB*$^{+}$	1%	4%	1626
Δ*smc::hyg* Δ*scpA::acc(3)IV*	4%	14%	3589
Δ*smc::hyg* Δ*scpAB::acc(3)IV*	3%	17%	3148

[a]One hundred random spore chains were quantified and the percentage of anucleate and bilobed spores were determined. All spores were considered regardless of the compartment size.

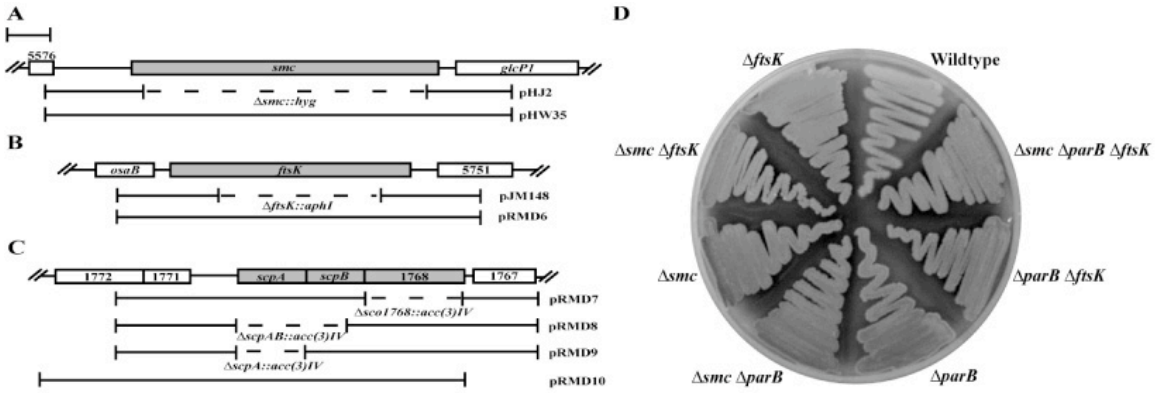

Figure 2.1 Physical maps of regions of chromosomal DNA containing *smc*, *ftsK* or *scpAB*. In each case, the flanking genes are in the same orientation as the genes of interest. Scale bar: 1 kb. (A) The *smc* region of the chromosome. *SCO5576* is predicted to encode an acyl phosphatase and *glcP1* is involved in glucose uptake. For mutagenic plasmid pHJ2, the region deleted and replaced with a hygromycin-resistance gene is shown (dashed line). The insert in complementation plasmid pHW35 is shown (bracketed line). (B) The *ftsK* region of the chromosome. *osaB* is part of a two-component regulator involved in osmoadaptation and *SCO5751* is predicted to encode a hypothetical membrane protein. For mutagenic plasmid pJR148, the region of *ftsK* deleted and replaced with a neomycin-resistance gene is shown (dashed line). The insert in complementation plasmid pRMD6 is shown (bracketed line). (C) The *scpAB* region of the chromosome. *SCO1768*, *SCO1771* and *SCO1767* are predicted to encode a pseudouridine synthase, a hypothetical protein found in actinomycetes and a possible DNA hydrolase, respectively. For mutagenic cosmids pRMD7-9, respectively, the regions of *SCO1768*, *scpA* and *scpAB* deleted and replaced with an apramycin-resistance gene are shown (dashed lines). The insert in complementation plasmid pRMD11 is shown (bracketed line); the DNA insert ends 1 base after *SCO1768*. (D) Single, double and triple mutant strains macroscopically resemble wild type. Strains were grown on MS

agar for 4 days at 30°C. Mutant strains formed a robust, grey pigmented, aerial mycelium, with no obvious growth or developmental delays. Strains M145 (wild type), J2537 (Δ*parB*), JM148 (Δ*ftsK*), HJ2 (Δ*smc*), RMD3 (Δ*smc* Δ*ftsK*), RMD4 (Δ*smc* Δ*parB*), RMD5 (Δ*parB* Δ*ftsK*) and RMD6 (Δ*smc* Δ*parB* Δ*ftsK*) are shown.

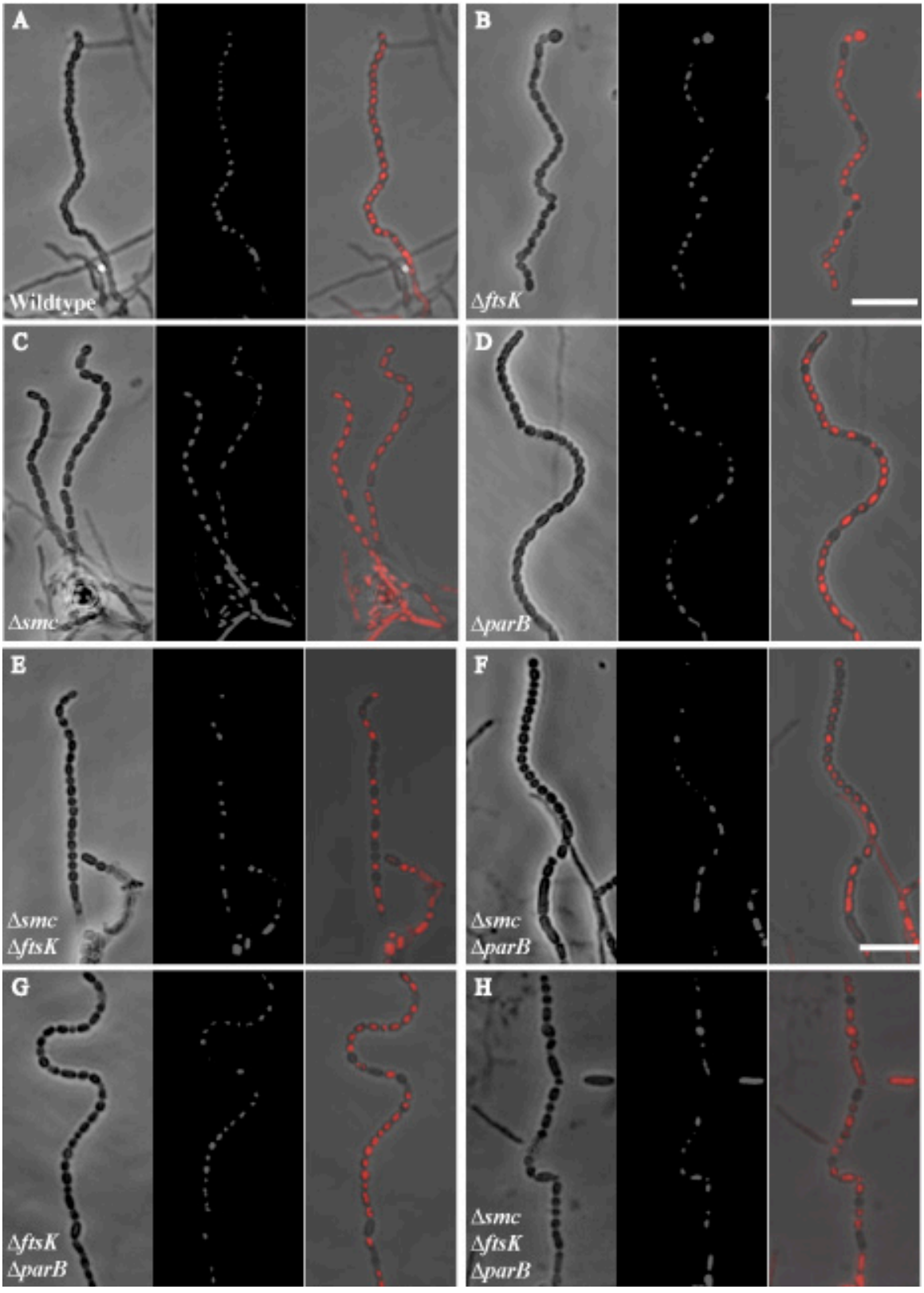
A
Wildtype
B
ΔftsK
C
Δsmc
D
ΔparB
E
Δsmc
ΔftsK
F
Δsmc
ΔparB
G
ΔftsK
ΔparB
H
Δsmc
ΔftsK
ΔparB

Figure 2.2 Confocal microscope images showing sporulation and DNA segregation phenotypes of wild type, single, double and triple mutant strains. Four-day-old cultures grown on MS agar were sampled on coverslips, fixed and stained with propidium iodide (red). In each panel, phenotypes of representative aerial hyphae are shown as phase-contrast and fluorescent image pairs, followed by a merged image. (A), M145 (Wild type). (B) JM148 (Δ*ftsK*). (C) HJ2 (Δ*smc*). (D) J2537 (Δ*parB*). (E) RMD3 (Δ*smc* Δ*ftsK*). (F) RMD4 (Δ*smc* Δ*parB*). (G) RMD5 (Δ*parB* Δ*ftsK*). (H) RMD6 (Δ*smc* Δ*parB* Δ*ftsK*). Scale bar: 7 μm.

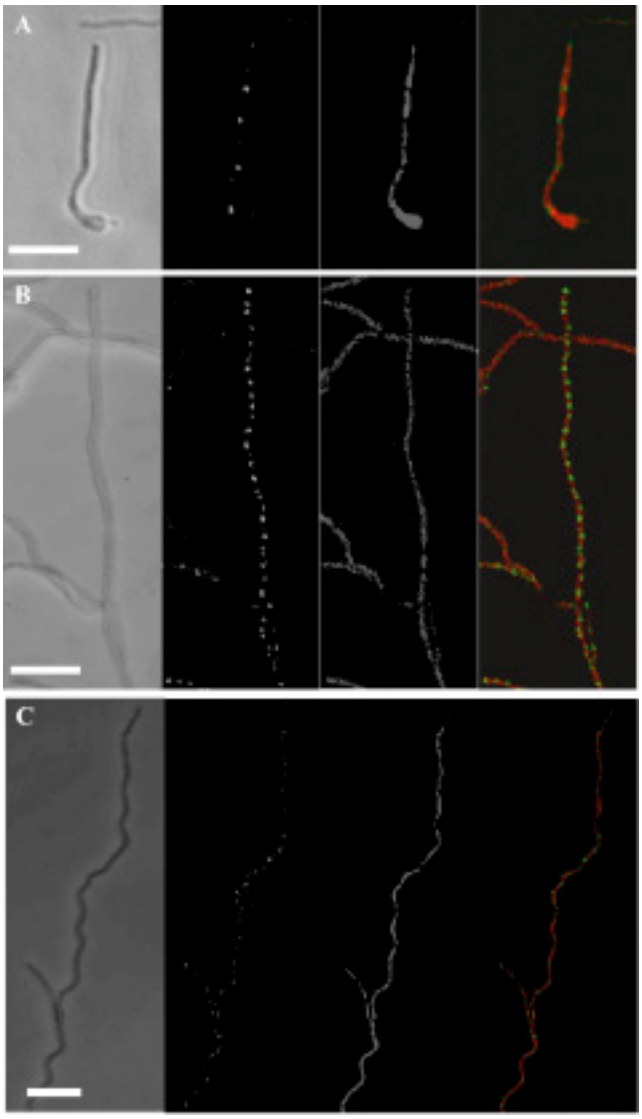

Figure 2.3 FtsK-EGFP and SMC-EGFP fusion protein localization in vegetative and reproductive hyphae. Coverslips were inoculated and grown for 1-3 days on MS agar, fixed and stained with propidium iodide. In each panel, representative hyphae are shown as phase-contrast and fluorescent images observed by scanning laser confocal microscopy. Left to right, panels contain phase-contrast, EGFP, DNA and a merge of the two fluorescent images. (A) FtsK-EGFP localized in a vegetative filament newly emerging from a spore (lower right, 1 day of growth). (B) FtsK-EGFP localized in a regular pattern in predivisional aerial filament just prior to prespore formation (long,

central filament oriented with long axis of panel, 2 days of growth). (C) The SMC-EGFP fusion protein had an irregular punctuate pattern of localization in a predivisional aerial filament (2-3 days of growth). SMC-EGFP foci are not uniformly distributed nor associated with every nucleoid. Scale bar: 7 μm.

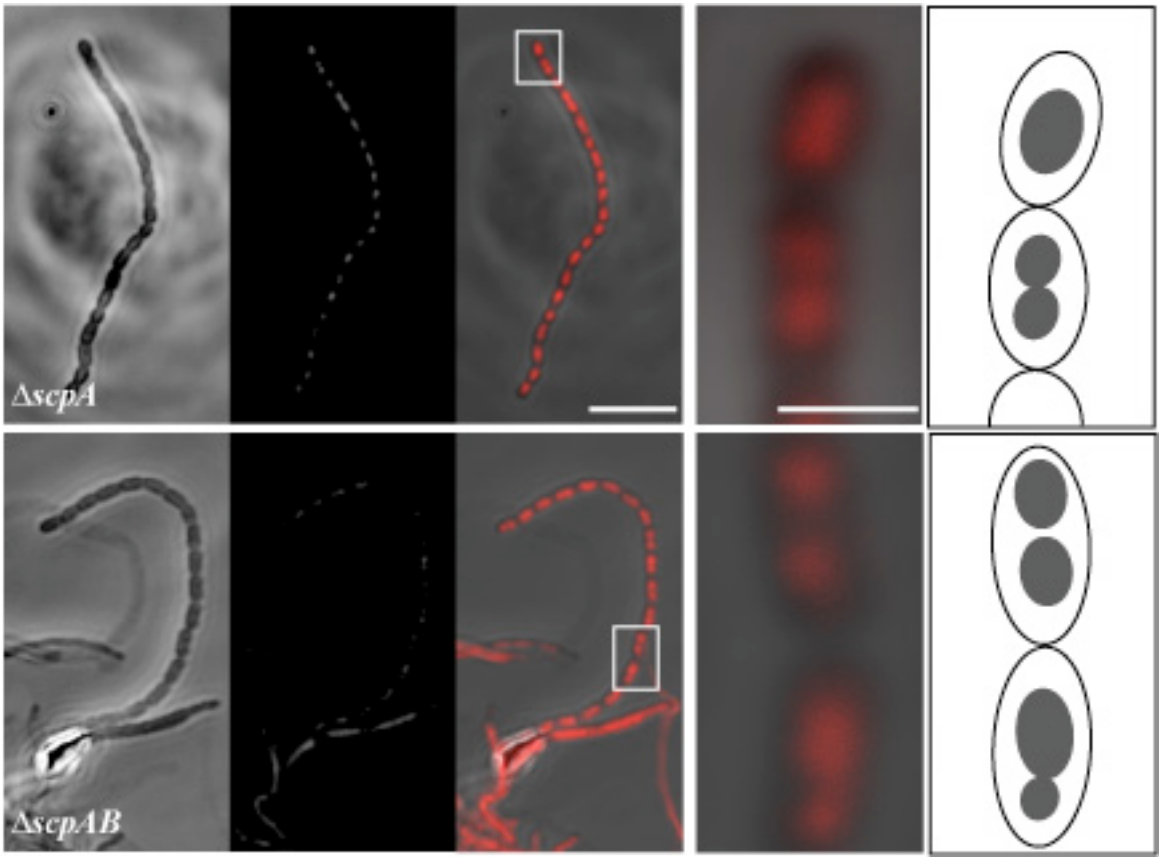

Figure 2.4 The *scpA* and *scpAB* mutants have a bilobed nucleoid phenotype.

Phase contrast and fluorescent images of the same fields were taken by scanning laser confocal microscopy. Coverslips were inoculated and grown for 1-3 days on MS agar, fixed and stained with propidium iodide. In each panel, phenotypes of representative aerial hyphae are shown as phase-contrast and fluorescent image pairs, followed by a merged image. For the merged images, the areas in the white boxes are enlarged and a cartoon interpretation is provided. (**A**) Images of Δ*scpA* (RMD18) showing spores with DNA appearing bilobed, unlike wild type spores. (**B**) Images of Δ*scpAB* spores (RMD19) also appearing to have a bilobed DNA pattern. Scale bar: 7 μm.

Chapter 3

DNA architecture and ploidy of *Streptomyces coelicolor* spores

ABSTRACT

Many prokaryotes have a single, circular genome, which is segregated faithfully each time the cell undergoes division. In contrast, for *S. coelicolor*, condensation and partitioning of the large, linear genome must occur accurately in the aerial filaments so that the multiple adjacent single-cell prespore compartments simultaneously receive a copy of the chromosome. The haploid nature of *S. coelicolor* spores has long been accepted. However, we report that our DNA intensity analysis of wild type *S. coelicolor* spores suggest a diploid nature. In addition, ParB-EGFP was used as an indirect marker of the origin of replication (*oriC*) to show that two copies of the origin region of the chromosome are localized late in the maturation of each spore compartment, one at each pole of the spore. We identified a paralogous partition gene which plays a role, directly or indirectly, in positioning *oriC* in the spore late in development. Deletion of the *parA2* gene appears to cause mislocalization of the *oriC* region late in spore development, as visualized indirectly using ParB-EGFP. The localization of ParA2 was determined using a translational EGFP fusion, which revealed that this protein forms a band or bands near the upper end of the apical compartment in predivisional aerial filaments with increased diffuse fluorescence toward the tip. The advantage of having two copies of the

chromosome per spore could be to ensure survival, as this bacterium is typically found in the hostile soil environment.

INTRODUCTION

Most prokaryotes reproduce to form haploid daughter cells, but several have been discovered that produce diploid spores. *Myxococcus xanthus, Bacillus megaterium* and *B. cereus* produce spores that contain two copies of the chromosome (Tzeng & Singer, 2005; Woese, 1958). While it is believed that *Streptomyces coelicolor* spores are haploid, evidence of this has not been well documented.

S. coelicolor is a Gram positive, filamentous bacterium with a large 8.7 Mbp linear chromosome (Bentley *et al.*, 2002). Its life cycle resembles certain filamentous fungi and consists of two growth phases, vegetative and reproductive. Vegetative mycelial growth commences with spore germination and the production of a branched network of hyphae, or mycelium, which is macroscopic. The vegetative mycelium gives rise to reproductive aerial filaments, which grow away from the colony surface. The syncytial aerial filaments synchronously form up to 100 septa, while also segregating and condensing chromosomes simultaneously. The rod-shaped prespore compartments further mature into ovoid-shaped spores. They have solved a strategy for progeny distribution; spores produced in the reproductive phase are dispersed to new locations.

Our previous genetic work focused on how dozens of copies of the large, linear chromosome of *S. coelicolor* were simultaneously segregated and condensed into prespore compartments of aerial filaments. The nucleoid morphology of one mutant strain revealed bilobed DNA architecture in a substantial fraction of its spores (Dedrick *et al.,* 2009), which prompted further investigation into the ploidy of wild type spores. Our

current microscopic analysis suggests that wild type *S. coelicolor* spores are diploid. Additional analysis of six different streptomycetes suggests that this is a common feature for spores produced by species of this genus. Furthermore, the orientation of the DNA in the spore was analyzed. Indirect studies of chromosome arrangement within spores, using ParB-EGFP, suggest that each origin of replication (*oriC*) localizes to the spore poles late in development. Previously, it was found that ParA positions ParB-EGFP foci in early predivisional aerial filaments (Jakimowicz *et al*., 2007); therefore, we searched the genome for additional partition gene homologues which might be involved in post-septation DNA movement. *S. coelicolor* contains a second homolog for *parA*, which we named *parA2*. A *parA2* mutant appears to be unable to properly organize the *oriC* regions to the poles of the spores, as judged by ParB-EGFP foci. How this organism is able to synchronously segregate and condense two copies of its large chromosome each within as many as 100 spores increases the complexity of an interesting problem. We propose a new model where origins are "hand-cuffed" at septation with single, large ParB-EGFP focus located centrally in the prespore compartment; ParA2 directly or indirectly separates this large ParB-EGFP focus into smaller foci in bipolar locations.

MATERIALS AND METHODS

Bacterial Growth and Media

Bacterial strains used in this study are listed in Table 3.1. YEME (liquid), MS (agar), R2YE (agar) or MM (agar with 0.5% glucose (w/v)) were used for growth of *S. coelicolor* at 30°C (Kieser *et al*., 2000; McCormick *et al*., 1994). Standard procedures for protoplast preparation and fusion were used (Kieser *et al*., 2000). The multiple auxotrophic *S. coelicolor* strain 2709 was used as a donor strain for conjugation to

introduce plasmids into mutants. Final concentrations of antibiotics used for *S. coelicolor* were: apramycin, 25 μg ml^{-1}; hygromycin, 200 μg ml^{-1}; kanamycin, 160 μg ml^{-1}; and thiostrepton, 50 μg ml^{-1}. The viability of the wild type and certain mutant strains was determined using the average of at least two independent spore preparations (growth on MS) analyzed from duplicate serial dilutions by direct count using phase-contrast microscopy and viable count after 3 days of incubation on glucose MM; heat resistance was tested in 0.85% saline at 65ºC for 15 min. For DNA content analyses, all *Streptomyces* species were grown on MS and spores were harvested after 3 days at 30°C. *M. xanthus* strain DK101, a gift from M. Singer (UC Davis), was grown at 33ºC on CTTYE (casitone/Tris/yeast extract) liquid and solid media, which contained 1.5% agar (Bretscher & Kaiser, 1978). Development of *M. xanthus* was completed as reported (Tzeng & Singer, 2005). Briefly, cells were grown in CTTYE medium until an A_{600} of 0.5 was reached. The cells were concentrated 10 to 1, spotted onto TPM agar (Hagen *et al*., 1978) and incubated at 33ºC for 5 days before harvesting. *B. subtilis* PB2 and *B. megaterium* QMB1551 were grown at 37ºC on Luria broth containing 1.5% agar and sporulation was induced by growth in DSM medium (liquid) (Nicholson & Setlow, 1990) for 24 h.

E. coli strain TG1 was used for standard plasmid manipulation (Sambrook *et al*., 1989). Strain BW25113 containing λ Red plasmid pIJ790 (Gust *et al*., 2004) was used for *in vivo* construction of *egfp* fusions and insertion-deletion mutations. In order to circumvent the methyl-specific restriction system of *S. coelicolor*, *E. coli* strain ET12567 (*dam dcm hsdM*) was used to prepare unmodified plasmid and cosmid DNA (MacNeil *et al*., 1992). Strain ET12567/pUZ8002 was used as a donor strain for interspecies

conjugation. *E. coli* strains were grown at 37ºC in Luria broth. Final concentrations of antibiotics used for *E. coli* were: ampicillin, 100 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; apramycin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; and kanamycin, 50 μg ml^{-1}.

Plasmids and general DNA techniques

Plasmids used in this study are listed in Table 2. Plasmid preparations were obtained using QIAprep Spin Miniprep Kit (Qiagen). *S. coelicolor* total DNA preparations were obtained using the Wizard Genomic DNA Purification Kit (Promega). DNA restriction and modifying enzymes (New England Biolabs), as well as *Taq* (Promega) and *Pfx* (Invitrogen) DNA polymerases, were used according to manufacturers' instructions. Redirect technology (Gust *et al*., 2004) was used for λ Red-mediated recombination using mutagenic linear DNA cassettes in *E. coli*.

Isolation of deletion-insertion mutants

Insertion-deletion mutations in *parA2* (*SCO1772*), *SCO1771*, and *geoA* (*SCO6073*) of *S. coelicolor* were constructed *in vivo* using *E. coli* λ Red-mediated recombination and cosmids SCI51 and SC9B1, respectively. In cosmid derivatives pRMD12 (Δ*parA2::acc(3)IV*), pRMD13 (Δ*SCO1771::acc(3)IV*), pRMD14 (Δ*geoA::acc(3)IV*) and pRMD15 (Δ*geoA::hyg*), the *parA2, SCO1771,* or *geoA* genes were replaced by a cassette containing a resistance gene and *oriT* flanked by FRT sites (Gust *et al*., 2004). The resistance gene cassettes were obtained from pIJ773 and pIJ10700 and used as template for PCR. Oligonucleotides (Table 3.3) were used for PCR to add homology to the specific cassette with either *parA2, SCO1771,* or *geoA*. The insertion-deletion mutations in each of the cosmids were introduced into the chromosome by conjugation, selecting either for apramycin or hygromycin resistance, and screening

for kanamycin sensitivity. Strains RMD25 (Δ*parA2::acc(3)IV*), RMD26 (Δ*SCO1771::acc(3)IV*), RMD31 (Δ*geoA::acc(3)IV*) and RMD32 (Δ*geoA::hyg*) were isolated and verified by Southern blot hybridization or diagnostic PCR. The Δ*geoA* mutation isolated for this study was identical to the previously published one, which had no discernable phenotype other than the cessation of geosmin production (Gust *et al*., 2003).

Strain RMD29 ((Δ*parA2::acc(3)IV parB-egfp*) was constructed by conjugating pRMD12 into J3310 (*parB-egfp*), selecting for apramycin resistance and kanamycin sensitivity.

Southern blot hybridization analysis and diagnostic PCR

A 4.8 kb *Xba*I fragment from SCI51 was used as a probe for verification of *parA2* and *SCO1771* deletions. A 5.8 kb band was obtained for wild type; 2.4 kb and 3.4 kb bands were obtained for Δ*parA2*; 2.7 kb and 3.8 kb bands were obtained for Δ*SCO1771*. Hybridization reactions were performed at 65 ºC using buffer that contains 5% SDS (Virca *et al*., 1990). Nylon membranes (Hybond-N, Amersham) were used as a solid support and probes were non-isotopically labeled before immunological detection (DIG DNA Labeling and Detection Kit, Boehringer Mannheim).

The *geoA* mutants were verified by PCR using oligonucleotides geoA20Fwd and geoA20Rev (Table 3.3). A 2.5 kb band was amplified from wild type, a 1.6 kb band was amplified for the Δ*geoA::acc(3)IV* and a 1.8 kb band was amplified for the Δ*geoA::hyg*.

Genetic complementation

Complementation of RMD25 (Δ*parA2::acc(3)IV*) and RMD29 (Δ*parA2::acc(3)IV parB-egfp*) was completed using pRMD11 (Table 3.2), a previously

described SCP2* derived plasmid (Dedrick *et al*., 2009). pRMD11/2709, was mated with RMD25 and RMD29 selecting for thiostrepton resistance and prototrophy. Transconjugants were tested for the presence of the antibiotic-resistance gene marking the chromosomal mutation. Because pRMD11 contains five *S. coelicolor* genes, including *parA2*, but not the complete intergenic region upstream of *parA2*, another complementation plasmid was constructed. First, the *E. coli* λ Red recombination system was used to replace the apramycin resistance gene with a spectinomycin-resistance gene on the backbone of pSET152, resulting in plasmid pSpc152. The spectinomycin resistance fragment was isolated from pKP2, a subclone of pHP45Ω, (K. Pevey & J.R.M., unpublished results). Oligonucleotides 2pUC61Spc and pSET60Spc were used to add homology to the *aadA* fragment. A 3.6 Kb *Bgl*II fragment of SCI51, containing *SCO1771* and *parA2* as the only complete genes, was cloned into the *Bam*HI site of pSpc152 creating pRMD16 (Table 3.2). pRMD16 will be conjugated into RMD25 and RMD26, where it will integrate into the ΦC31 *attB* site in the chromosome, creating RMD33 and RMD34, respectively.

Construction of ParA2-EGFP

A *parA2-egfp* translational fusion was constructed by *in vivo* recombination. Cosmid H24*parB-egfp* (Jakimowicz *et al*., 2005) was used as the template for PCR using oligonucleotides which added 40 bases of *parA2* homology to the *egfp-acc(3)IV* cassette (Table 3.3). The PCR product was introduced into SCI51 selecting for apramycin-resistant transformants creating I51*parA2-egfp*. The gene fusion junction with a linker peptide (LPGPE) and *egfp* sequence was verified. This cosmid was conjugated into wild type strain M145, selecting for apramycin-resistance and screening for kanamycin

sensitivity. A transconjugant containing the fusion as the only copy of *parA2* was named RMD31.

ParB Localization

Strain J3310 (*parB-egfp*, unmarked) was a gift from D. Jakimowicz (Jakimowicz *et al*., 2005).

Microscopy

Strains were characterized by laser scanning confocal microscopy using a Leica SP2 microscope equipped with a phase-contrast 100X oil objective (1.40 NA) and 488- and 543-nm lasers. Myxospores were purified from other cells in the fruiting body as previously reported (Tzeng & Singer, 2005). *S. coelicolor* spores were collected in sterile water and centrifuged at 5000 xg for 5 min. The supernatant was removed and spores were resuspended in 500 µl of PBS containing 4% paraformaldehyde. For DNA intensity profile measurements, propidium iodide stained spores were resuspended in 50% glycerol and applied to Poly-D-Lysine (Sigma) coated slides. The DNA intensity profiles were collected with a constant laser intensity and obtained using the ellipse option of the stack profile tool using Leica software version 2.61 Build 1537. The average intensity readings for each strain were collected and listed in Table 3.4 and 3.5. For analysis of phenotypes of Δ*parA2* mutant and EGFP localization, sterile coverslips were embedded at a 45° angle in MS agar, inoculated on the obtuse angle, and incubated for the indicated lengths of time. Coverslips were removed and cell material was fixed at room temperature for 10 min using a phosphate buffered saline (PBS) solution containing 4.375% glutaraldehyde and 0.028% paraformaldehyde. The fixed coverslips were washed twice with PBS, allowed to air dry, and mounted in 50% glycerol containing 0.1% of the cell membrane

specific stain FM-4-64 (Molecular Probes), or treated with 10 mg/ml lysozyme (in TGE) for 2 min, and mounted in 50% glycerol containing 0.1% of the cell wall stain Wheat Germ Agglutinin-Texas Red (Molecular Probes) (Schwedock *et al*., 1997). The *parA2-egfp* strain RMD31 was processed using a rabbit polyclonal GFP antibody (ABCAM-ab6556) to intensify the signal (gift from S. Watkins). Coverslips were inoculated, fixed, and treated with lysozyme as above. They were then washed with PBS, blocked with BSA (2% in PBS) for 5 min at room temperature, treated with anti-EGFP antibody (1:500 dilution in BSA) and incubated overnight at 4°C. The antibody solution was removed and cells were washed 10 times with PBS and mounted in 50% glycerol. Images were adjusted for brightness and contrast using Adobe Photoshop and assembled in Adobe Illustrator.

RESULTS

Quantitation of DNA content of *S. coelicolor* spores

In a previous genetic study, we isolated mutants for genes encoding *smc* associated proteins (ScpA and ScpB). *scpA* and *scpAB* mutants produced spores with altered nucleoid morphology (Dedrick *et al*., 2009). Wild type spores stained with propidium iodide (PI) show a single, condensed nucleoid region that completely fills the spore compartment. Interestingly, the Δ*scpA* and Δ*scpAB* mutants of *S. coelicolor* produced a substantial fraction of spores with a "bilobed" nucleoid morphology (Dedrick *et al*., 2009). This phenotype was intriguing and suggested that either haploid spores contain two condensed domains of the chromosome, which are usually held together, or alternatively, that the mutant spores could be diploid. This result raised the question as to whether wild type spores might be diploid.

In order to determine the ploidy of wild type spores, fixed spores were RNase treated and stained with propidium iodide. Confocal microscopy was used to take a series of optical Z-sections through a spore, generating a stack. The area of interest in the stack was quantified using the stack profile tool. The intensity of the PI fluorescent signal for the nucleoid was measured. This method is similar to flow cytometry, which is frequently used for similar measurements, yet is more sensitive and accurate.

In order to determine the DNA content in *S. coelicolor* spores, we created a standard relating PI-staining intensity to genome size and ploidy (Table 3.4). *M. xanthus* was recently shown to produce diploid spores using flow cytometry and FISH (Tzeng & Singer, 2005). *M. xanthus* has a large 9.14 Mb genome (Goldman *et al*., 2006), similar to the 8.7 Mbp genome of *S. coelicolor* (Bentley *et al*., 2002), and was used as a control for these experiments. We used the same two control species as the *M. xanthus* study. Wild type strain, PB2, of *B. subtilis* was used as a control for quantitation; the chromosome size of the haploid spores is known to be 4.2 Mb (Kunst *et al*., 1997; Woese, 1958). *B. megaterium* spores are diploid and have a 5.1 Mb genome (Hauser & Karamata, 1992; http://www.bios.niu.edu/b_megaterium/). This method assumes that PI stains equally or is similarly accessible to all DNA in these very different types of spores, which may not be the case (Table 3.4).

The DNA intensity reading of *B. megaterium* spores suggested that they contained approximately twice as much DNA as the *B. subtilis* spores, which is expected according to their known genome sizes and ploidy. The intensity readings of *M. xanthus* spores suggested that they contained about twice the amount of DNA than *B. megaterium* spores. This data suggested that the DNA intensity method used here to quantitate

genetic material was accurate and various fixed and processed spores were susceptible to stain. As evidence, the staining intensities (arbitrary units with a constant laser intensity) for the control species were similar, ranging from 11.2-15.7 units/Mb (Table 3.4).

If *S. coelicolor* spores were diploid, it was anticipated that their DNA intensity measurement would be similar to that of *M. xanthus* spores. If *S. coelicolor* spores were haploid, it was expected that their DNA intensity reading would be similar to that of *B. megaterium* spores. Using this method, the data are consistent with the interpretation that *S. coelicolor* spores were diploid because the DNA intensity reading was more similar to that of *M. xanthus* (238 vs. 205), rather than *B. megaterium* (238 vs. 114) (Table 3.4). Furthermore, for *S. coelicolor*, the intensity unit per Mb was 13.7 units/Mb if spores were diploid, which is within the range measured for the control species (11.2-15.7), and 27.4 units/Mb if haploid, which falls well outside the control range. In all, the data are consistent with *S. coelicolor* spores being diploid.

We also tested spores from six additional *Streptomyces* species and all produced data that was similar to the intensity readings of *S. coelicolor* spores (Table 3.5), consistent with the interpretation that many members of this genus produce diploid spores.

In the laboratory, *S. coelicolor* is grown on nutritious media to provide robust growth and sporulation. In the soil, conditions vary; therefore, in order to determine if diploid spores are only produced in a nutrient-rich environment, we determined the DNA intensity of spores produced on minimal glucose medium. The medium from which the spores were harvested did not affect their DNA intensity measurements (data not shown).

ParB localization reveals unexpected DNA dynamics late in development

ParB binds to 24 *parS* sites in the chromosome within 200 kb *oriC* to form a large nucleoprotein complex that is believed to help position and partition the chromosome during DNA segregation (Jakimowicz *et al*., 2002 & 2005). ParB-EGFP localization can therefore be used to indirectly visualize the origin region of the chromosome and reveal *oriC* DNA location, as has been used in other organisms (Lee & Grossman, 2006). Early in the lifecycle, ParB-EGFP localizes irregularly in vegetative hyphae, suggesting that chromosomes are not highly organized in these filaments (Jakimowicz *et al*., 2005). However, in reproductive aerial filaments, ParB-EGFP foci assemble as bright, regularly-spaced foci in between where septa will form, helping to ensure that each prespore compartment receives chromosomal material (Jakimowicz *et al*., 2005). We also observed these vegetative and aerial hyphae localization patterns of ParB (Figure 3.1A), but were interested in the location of the origin region of the chromosome in the maturing spore.

In a mycelium, all of the aerial hyphae are not developing synchronously, but at any time, different developmental stages are represented in the population of hyphae. It was previously suggested that ParB foci dissipated and ParB was degraded once septation was complete (Jakimowicz *et al*., 2005). After specifically searching for examples of aerial hyphae at a stage just after septation, present in an infrequent percentage of hyphae, we observe two well-separated ParB-EGFP foci in prespore compartments beginning to metamorphose (Figure 3.1). This suggests that after septation ParB foci do not immediately disappear and actually provide evidence to further support the diploid nature of spores. Fluorescence microscopy analysis revealed that ParB foci are still apparent after septation occurs, when rod-shaped prespores further mature and begin to round.

The process is synchronous as most spores within the same hypha have two ParB foci. At this late stage of development, ParB foci are observed at opposite poles of the spore in a specific "North" and "South" location (Figure 3.1). We propose that the large centrally located ParB-EGFP foci in prespore compartments consists of two "handcuffed" copies of *oriC*. This central localization may provide a way to "capture" both copies of the chromosome in a single-cell compartment. Then, after septation, the two chromosomes are separated, sending sister *oriC* regions to an opposite pole ("North" and "South") of the maturing spore, late in development. This stage of development must be very short lived as aerial filaments with ParB foci at opposite poles are infrequently observed and easily missed. This observation suggests a specific location of the *oriC* region of the DNA inside the spore.

Previously, in *S. coelicolor*, fluorescence *in situ* hybridization (FISH) was used to determine that the ends of the linear chromosome co-localize in vegetative mycelia, suggesting that the ends may be held together (Yang & Losick, 2001). To directly visualize the location of two *oriC* regions in *S. coelicolor* spores, FISH would provide direct evidence. Unfortunately, this technique was challenging to perform on *S. coelicolor* spores. One hybridization experiment using an origin probe revealed several examples of spores with two foci, one at each pole of the spore (Appendix III). Despite many repeated attempts, the result could not be reproduced.

Nonetheless, the ParB-EGFP localization pattern suggests the polar location of the *oriC* region of the chromosome in the spore. This result is consistent with the unicellular bacteria, like *E. coli* and *C. crescentus*, which contain one chromosome, and localize *oriC* near one pole of the cell (Wang *et al*., 2005; Jensen & Shapiro, 1999). This

result is also consistent with *M. xanthus* FISH data showing each *oriC* localized at opposite poles of the diploid spore (Tzeng and Singer, 2005).

Identification of a second *parA* gene

In *S. coelicolor* and in other bacteria with a *par* system, ParB works in conjunction with ParA, encoded by a gene that is located immediately adjacent to *parB*. It was suspected that ParA provides the energy for the movement of ParB segregation complexes (Leonard *et al*., 2005). ParA (referred to hereafter as ParA1) of *S. coelicolor* forms helical structures in aerial hyphae and is required for the proper assembly of bright, centrally-located ParB complexes (Jakimowicz *et al*., 2007). Bacterial two-hybrid analysis indicated that ParA1 and ParB interact with each other (Jakimowicz *et al*., 2007). Because the *parA1* deletion mutant produced anucleate spores (26%), suggesting a role for the protein product in DNA segregation (Jakimowicz *et al*., 2007), we decided to explore the *S. coelicolor* genome for other potential *parA*-like genes that could possibly play a role in the newly observed process of separating the *oriC*-ParB complexes to opposite poles of the spore. Upon BLAST analysis of the ParA1 amino acid sequence, SCO1772 (referred to hereafter as ParA2) was found to have 41% (122/296) homology to ParA1 (Figure 3.2). *parA2* is 1,023 bp and is predicted to encode a 340 amino-acid protein. This second gene was likely overlooked as the *parA1 parB* region was sequenced prior to the genome project and *parA2* is not annotated as *parA*. *parA2* is unpaired and located one gene upstream of *scpAB*, whose gene products associate with the segregation protein SMC. The genome sequences of *S. avermitilis*, *S. griseus* and *S. scabies* reveal that all three have two *parA* genes (*parA1* paired with *parB* and an unpaired *parA2*). It seems that this is not true for all Actinomycetes, as *Frankia,*

Mycobacterium tuberculosis, Tropheryma whipplei, Corynebacterium diphtheriae, and *Rhodococus* do not have two *parA* genes (Figure 3.3).

ParA2 is required to establish polar *oriC* location in spores

Using the λ Red recombination system and cosmid I51, a deletion-insertion mutation was isolated *parA2* and the mutation was introduced into the chromosome resulting in strain RMD25 (Δ*parA2::acc(3)IV*), which did not have a growth rate defect and macroscopically resembled wild type. Because *parA2* is a homolog of a partition gene, DNA segregation was analyzed during spore formation using the nucleic acid stain propidium iodide. DNA segregation was mildly affected in aerial hyphae of the Δ*parA2* mutant (3% of 1633 total spores compared to 1% for wild type and 26% for Δ*parA1* (Jakimowicz *et al*., 2007)). Because previous data suggested that ParA1 interacts with ParB and affects its localization (Jakimowicz *et al*., 2007) we predicted that ParA2 might also play a role with ParB localization. ParB-EGFP localized normally throughout the lifecycle of the Δ*parA2* mutant, until the maturation of spores. In strain RMD29 (Δ*parA2::acc(3)IV parB-egfp*), large ParB foci aggregate normally to the center of early prespore compartments, but as the prespore matures the orientation of the foci is disrupted. Synchronously, in multiple adjacent spores, the ParB foci were seen drifting in more lateral positions to the "East" and "West" of the spore, instead of the normal "North" and "South" poles (Figure 3.4). We suggest that ParA2 may directly or indirectly correctly positions ParB foci (and *oriC*) at the poles of the spore.

This switch in chromosome orientation, led us to question if viability of the spores was affected in the Δ*parA2* mutant compared to wild type. Viability assays in which direct count of the spores was compared to the production of colonies (CFU) on minimal

glucose agar plates was determined. We found that spore viability was not affected in the Δ*parA2* mutant compared to wild type *S. coelicolor* (each were approximately 30% viable). Also, the Δ*parA2* mutant spores were no more or less resistant to heat as compared with wild type (3-fold drop after 15 min at 65°C). Therefore, it seems that the disorientation of the DNA does not overtly affect spore germination or resistance to heat treatment.

The gene that encodes ParA2 is located immediately upstream of a gene encoding the hypothetical protein SCO1771 and they are predicted to be co-transcribed (Figure 3.2). Because the deletion of *parA2* might be polar on the expression of *SCO1771*, we constructed pRMD13 (Δ*SCO1771::acc(3)IV*) and introduced the mutation into both the wild type and a strain expressing ParB-EGFP. The *SCO1771* deletion-insertion mutant did not have an altered growth rate, DNA segregation or condensation, or ParB-EGFP localization (data not shown). We conclude that the phenotypic effects observed for the Δ*parA2* mutant in the strain expressing ParB-EGFP resulted from the deletion of *parA2*.

Genetic complementation experiments were completed to confirm that the phenotype observed was due to the deletion of *parA2* and not to an unlinked mutation. Strain RMD27 (Δ*parA2::acc(3)IV / parA2*$^{+}$) was isolated using pRMD11 (Table 3.2) and the phenotype was analyzed. ParB-EGFP localization in the complemented strain resembled wild type in that late in development most of the spores exhibited a "North" and "South" localization of the *oriC* regions. Our complementation data suggests that the defects observed in ParB-EGFP localization resulted, at least in part, from the introduced *parA2* insertion-deletion mutation (data not shown). At least one promoter, active in aerial hyphae, must be present on the DNA insert used for genetic complementation.

However, proper temporal and spatial expression may require that the genes be located in their native chromosomal locations.

Localization of ParA2

parA2 was replaced by *parA2-egfp* in the chromosome of the wild type strain. This strain (RMD31) macroscopically resembled wild type and produced normal spores, as observed by phase-contrast microscopy. Localization of ParA2-EGFP was analyzed at several points during the life cycle. In vegetative filaments, ParA2-EGFP was irregularly localized, suggesting no distinct pattern of localization. In predivisional aerial filaments, ParA2-EGFP had two distinct localization patterns. Some aerial filaments had a bright band or bands of ParA2 fluorescence near the upper ¼ of the apical compartment; increased diffuse fluorescence is seen from the band(s) toward the tip in these filaments (Fig. 3.4 A & B). Alternatively, some aerial filaments have punctate ParA2 fluorescence near the tip of the filament (Fig. 3.4 C & D). This could suggest that ParA2 localization is dynamic. ParA2-EGFP fluorescence was not observed in mature aerial filaments with spore compartments.

Genetic Proof of Diploid Spores

We attempted to construct a heterozygous spore in order to lend genetic support that *S. coelicolor* spores were diploid. By creating a deletion-insertion mutation in a nonessential gene, antibiotic resistance markers could serve as selection agents for heterozygotes. The same deletion mutation was made in two separate strains; the only difference being the antibiotic resistance marker replacing the gene. Protoplast fusion was used, selecting with both antibiotics, to isolate a heterozygous mycelium.

Geosmin (*SCO6073*) is the secondary metabolite produced by *S. coelicolor* that gives soil its earthly odor. *geoA* encodes a protein which is required for geosmin biosynthesis (Gust *et al*., 2003). Strain RMD31 was isolated with an apramycin resistance marker replacing *geoA* (Δ*geoA::acc(3)IV*) and strain RMD32 has a hygromycin resistance marker replacing *geoA* (Δ*geoA::hyg*).

Protoplasts of both RMD31 and RMD32 were prepared and diluted to similar density (by eye) and then mixed. Heterozygous colonies were selected using apramycin and hygromycin and were obtained at a low frequency. Potential heterozygous colonies were streaked onto MS plates containing both antibiotics. These potential heterozygotes appeared macroscopically sick, as they produced small and slow growing colonies that were heavily pigmented.

Six colonies were restreaked on MS media containing hygromycin and apramycin to verify their antibiotic resistant phenoytpes. However, only one out of six colonies was able to sporulate well when grown on both antibiotics. Spores of this candidate were collected, diluted, and plated on MS agar without antibiotics. Colonies were then replica-plated. This was completed to determine how many colonies could grow on one antibiotic, but not the other due to the replication and segregation processes of the chromosomes. Data are currently being collected.

DISCUSSION

S. coelicolor spores appear to be diploid

The discovery of a bilobed DNA phenotype in spores of the *scp* mutants led me to reconsider the strength of data relating to the ploidy of *S. coelicolor* spores. In the past, X-ray inactivation was the method used to determine spore ploidy. Using this method,

B. megaterium was found to have a 2-hit survival curve, suggesting that it produced diploid spores (Woese, 1957). X-ray inactivation was completed on *Streptomyces griseoflavus* spores, which supported the notion that spores were diploid (Saito & Ikeda, 1959). This study also analyzed *S. coelicolor* spores, but determined the results to be inconclusive based on the tailing of the survival curve. Because we have better microscopy abilities and molecular methods today, we decided to determine the ploidy of *S. coelicolor* spores.

Our novel findings suggest that *S. coelicolor* spores are diploid. Quantitation of the DNA content in wild type spores indicated that the amount of DNA was similar to that of a *M. xanthus* spore, which has a similar sized genome and is diploid. Each copy of *oriC* is located at an opposite pole of the myxospore (Tzeng & Singer, 2005). In a subset of hyphae containing chains of immature spores, ParB-EGFP localization suggested that the two origins within *S. coelicolor* spores also exhibited polar localization. The location of the chromosome termini for replication (*ter*) was also located at the myxospore poles, suggesting a unique orientation of DNA (Tzeng & Singer, 2005).

Movement of *oriC* to bipolar positions late in spore development

ParB-EGFP foci supported the interpretation that the spores were diploid because after septation and segregation of the chromosomes into spore compartments, the two *oriC* regions are indirectly observed to be separated to opposite poles of the spore. This process appears to be synchronous, as adjacent compartments in a hypha were all at a similar stage. This polar localization must involve the movement of each *oriC*, which are initially located centrally in the prespore compartment earlier in development. Is this

movement passive or active? It would be hard to imagine the movement of each *oriC* to opposite poles of the spore as a passive process. There is a distinct location to which each *oriC* moves and this could not be accomplished synchronously in all adjacent compartments through the random movement and capture of the DNA inside the spore. Aso, this force cannot be from replication, as the last round appears to occur before septation. Therefore, we believe this has to be an active process facilitated by proteins inside the spore. The first protein that was suspected to play a role in this movement was ParA1, which is coexpressed from an operon with ParB. ParA1 was shown in *S. coelicolor* to assemble into helical filaments inside predevelopmental aerial hyphae, which are believed to be dynamic, and thought to play a role in ParB-*oriC* positioning. Because ParA was of interest to us, we searched the genome for additional ParA encoding genes. Surprisingly, we discovered a second paralog not coupled with a second *parB* gene with 41% identity to ParA1. Deleting *parA2* did not have an effect on the development of this organism and had only a mild DNA segregation defect (3% compared to 1% in wildtype). The *parA2* mutation did change the origin localization in spore compartments, as visualized by ParB-EGFP localization. Instead of the typical bipolar localization of *oriC* in each spore, the two *oriC* were located more toward the sides of the spore. This suggested that the deletion of *parA2* was directly or indirectly disrupting deliberate chromosome orientation. Either ParA2 plays an active role in the movement of the *oriC* regions of each chromosome to the poles, or it stabilizes an interaction with a protein, for example ParA1, that facilitates this movement. It is possible that ParA1 could be involved if it functions twice in development.

Because ParA1 was found to assemble in helical filaments in predivisional aerial hyphae, we did not suspect that ParA2 might have a similar localization pattern because it was needed for post-septation. The ParA2-EGFP localization pattern did not reveal strong localization in helical filaments, but bands and otherwise diffuse fluorescence toward the tip of aerial hyphae. Perhaps ParA1 and ParA2 can copolymerize into the helical filaments formed mainly by ParA1. The predivisional expression of ParA1 may be higher than ParA2, which may be why the helical filaments are pronounced when ParA1-EGFP is visualized. We are currently testing the interaction of ParA2 with ParA1 and ParB using a bacterial two-hybrid system.

We propose a model that early in development, the previously reported large, central ParB-EGFP foci represent two "handcuffed" origins. This model would resemble those proposed for low-copy number plasmids in unicellular bacteria. This mechanism may be used to ensure that each prespore compartment receives genomic material. Then, as the spore matures, the large central ParB-EGFP foci are synchronously separated into two smaller polar foci (Figure 3.6).

Why would the spore position each *oriC* region at opposite poles of the spore? To determine if bipolar orientation contributed to spore viability, we analyzed spore fitness in the *parA2* mutant. Compared to wild type, there was no difference in viability in the *parA2* mutant, which suggested that orientation is not critical to the survival or germination of the spores under the conditions tested in the laboratory environment. In nature, spores are in more hostile soil environments, where they are subject to extreme weather conditions and predation. Perhaps this orientation is a more advantageous way

to store chromosomes safely inside the spore and to ensure at least one undamaged copy in the wild.

The bipolar movement of each *oriC* could be attributed to other actin-like proteins MreB or Mbl. In *B. subtilis*, *mreB* and *mbl* are required for correct bipolar positioning of *oriC* after DNA replication in vegetative cells (Herve *et al*., 2003). These proteins form helical structures and polymerize to push the newly replicated origin regions to the poles of the cell (Jones *et al*., 2001). *S. coelicolor* has homologues of both MreB and Mbl. MreB is not essential for vegetative growth, but is critical in the formation of the spore cell wall (Mazza *et al*., 2006). Preliminary experiments indicate that *mbl* may be dispensible in *S. coelicolor* (M. Hasipek, R.M.D. & J.R.M., unpublished data) and experiments to determine if ParB-EGFP foci are disturbed are underway. It would be telling to determine if each *oriC* still moved to opposite poles of the spore late in development without one, or both, of these genes. This experiment could help determine what other proteins contribute or influence the movement of *oriC* regions.

How is this movement different from the placement of *oriC* in unicellular bacteria?

In cells that divide by binary fission, such as *E. coli*, the newly replicated chromosomes are located at the septum and each is pulled in the opposite direction of the other; this movement is coordinated with replication. Thereby, each daughter cell receives one copy of the chromosome. In *S. coelicolor*, the method of DNA segregation is obviously not as simple, as a Δ*ftsk* Δ*smc* Δ*parB* triple mutant is viable and 90% of spores still received some genetic information with a fourfold drop in germination efficiency (Dedrick *et al*., 2009). We now suggest that some of the unevenness of DNA

staining seen in some of the segregation mutants, in our study and others, reflects that some spores might obtain only one chromosome, not partial chromosomes.

The method of DNA segregation in *S. coelicolor* has become more complicated. This organism must synchronously segregate two chromosomes into each prespore compartment in a developing aerial filament. Are the two chromosome copies held together during the entire life cycle of *S. coelicolor*? The large ParB-EGFP foci seen in the middle of prespore compartments, suggests that the two chromosomes are held together there with their origin regions colocalized. Then, once the spores are maturing, the large ParB foci are separated into two smaller foci and are sent to opposite poles of the spore. This movement is not due to simultaneous chromosome replication and segregation, like that in dividing *E. coli* cells.

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Table 3.1

Bacterial strains used in this study.

Strains	Genotype	Reference or Source
S. coelicolor A(3)2	wildtype *proA1 hisA1 argA1 cysD18 uraA1 strA1*	
2709	SCP1$^-$ SCP2$^-$	Kieser *et al.*, 2000
J3310	*parB-egfp,* unmarked	Jakimowicz *et al.,* 2005
M145	prototroph SCP1$^-$ SCP2$^-$	Kieser *et al.*, 2000
RMD25	Δ*parA2::acc(3)IV*	This study
RMD26	Δ*SCO1771::acc(3)IV*	This study
RMD27	Δ*parA2::acc(3)IV* / pRMD11	This study
RMD28	Δ*SCO1771::acc(3)IV* / pRMD11	This study
RMD29	Δ*parA2::acc(3)IV*, *parB-egfp* unmarked	This study
RMD30	*parA2-egfp acc(3)IV*	This study
RMD31	Δ*geoA::acc(3)IV*	This study
RMD32	Δ*geoA::hyg*	This study
RMD33	Δ*parA2::acc(3)IV* / pRMD16	This study
RMD34	Δ*SCO1771::acc(3)IV* / pRMD16	This study
S. albus G	wildytpe	Lab stock
S. griseus B2682	wildytpe	Lab stock
S. hygroscopicus ATCC53653	wildytpe	Lab stock
S. lividans 66 TK24	*str-6* SLP2$^-$ SLP3$^-$	Lab stock
S. parvulus JI2283	*str-1*	Lab stock
S. venezuelae 10712	wildytpe	C. Stuttard
M. xanthus DK101	wildtype	M. Singer
B. subtilis PB2	wildtype	M. Singer
B. megaterium QMB1551	wildtype	R. Losick

*Except for 2709, all other *S. coelicolor* strains are derived from M145.

Table 3.2

Plasmids and cosmids used in this study.

Plasmid	Description	Reference
SCI51	cosmid source of *parA2* (*SCO1772*)	Redenbach *et al*., 1996
SC9B1	cosmid source of *geoA* (*SCO6073*)	Redenbach *et al*., 1996
H24*parB-egfp*	*egfp* inserted in-frame at 3' of *parB* in cosmid SCH24, *acc(3)IV aphII*	Jakimowicz *et al*., 2005
I51*parA2-egfp*	*egfp* inserted in-frame at 3' of *parA2* in cosmid SCI51, *acc(3)IV aphII*	This study
pBluescript II SK (+)	standard cloning vector	Stratagene
pJRM10	bifunctional cloning vector, low-copy-number in *S. coelioclor, bla tsr*	McCormick & Losick 1996
pRMD10	4.8 kb *Xba*I fragment from SCI51 containing *parA2* cloned into similarly digested pBluescript	Dedrick *et al*., 2009
pRMD11	24 kb *Hin*dIII-*Spe*I fragment from pJRM10 cloned into similarly digested pRMD10	Dedrick *et al*., 2009
pRMD12	cosmid SCI51 containing Δ*parA2::acc(3)IV*, by *in vivo* recombination	This study
pRMD13	cosmid SCI51 containing Δ*sco1771::acc(3)IV*, by *in vivo* recombination	This study
pRMD14	cosmid SC9B1 containing Δ*geoA::acc(3)IV*, by *in vivo* recombination	This study
pRMD15	cosmid SC9B1 containing Δ*geoA::hyg*, by *in vivo* recombination	This study
pRMD16	3.6 kb *Bgl*II fragment of SCI51 cloned into *Bam*HI digested pSpc152	This study
pSET152	itegrating, non-replicative conjugative cloning plasmid	Bierman *et al*., 1992
pSpc152	pSET152 with *acc(3)IV* replaced by *aadA*	This study

Table 3.3

Oligonucleotides used in this study.

Oligonucleotide	Sequence	Application
parA2Fwd	AGCACACATGAGTATGGATGGCCAACACGTGAAC GCCATGATTCCGGGGATCCGTCGACC	Construction of Δ*parA2*
parA2Rev	CTCACTCGGCGTGACACCGGGCGAGCACCTCCCT GGCGAGTGTAGGCTGGAGCTGCTTC	Construction of Δ*parA2*
parA2egfpFwd	CCAGCTCGCCAGGGAGGTGCTCGCCCGGTGTCAC GCCGAGCTGCCGGGCCCGGAGCTG	Construction of *parA2-egfp*
parA2egfpRev	CCTGTCGTACGGAAGAGTTCGTCGGCCCCCGGCA GACTCACATATGTAGGCTGGAGCTGC	Construction of *parA2-egfp*
1771up60	AGTGAGTCTGCCGGGGGCCGACGAACTCTTCCGT ACGACAATTCCGGGGATCCGTCGACC	Construction of Δ*sco1771*
1771down59	GGGCGTCGTTCGAGGTCATGGCCCCCGCAGGCTA CCGCTATGTAGGCTGGAGCTGCTTC	Construction of Δ*sco1771*
geoA59Fwd	CGGCGGATGCGGTCGAAGAGCCCTGGGTAGGGCC GGGCCATTCCGGGGATCCGTCGACC	Construction of Δ*geoA*
geoA58Rev	CGAGCCACGAAAGAGTGAGACTGAACGTCCGTCA GCGCGTGTAGGCTGGAGCTGCTTC	Construction of Δ*geoA*
geoA20Fwd	GGTCAGGAGCTGAGAAGGAG	PCR verification of Δ*geoA*
geoA20Rev	GCAACGCTGTCTGTTCCATG	PCR verification of Δ*geoA*
2pUC61Spc	TTGGTCATGAGATTATCAAAAAGGATCTTCACCT AGATCCGTGACGCACACCGTGGAAACG	Construction of pSpc152
pSET60Spc	CGGTGAGTTCAGGCTTTTTCATATCTCATTGCCC CCGGACGTTCGGCCCGCGCTTCCTCG	Construction of pSpc152

Table 3.4

Quantitation of DNA content of *S. coelicolor* and several spore formers of known ploidy and genome size.

	B. subtilis	*B. megaterium*	*M. xanthus*	*S. coelicolor*	
Genome Size (Mb)	4.2	5.1	9.1	8.7	
Ploidy	1N	2N	2N	if 1N	if 2N
Intensity Units/Mb DNA	15.7	11.2	11.3	27.4	13.7
Average Intensity Units*	66	114	205	238	
Standard Deviation	±16	±25	±17	±7	

*For each strain analyzed, 50 spores were measured. Intensity units are arbitrary.